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(54) Title: SCREENING AND THERAPY FOR LYMPHATIC DISORDERS INVOLVING THE FLT4 RECEPTOR TYROSINE KINASE (VEGFR-3)		
(57) Abstract <p>The present invention provides materials and methods for screening for and treating hereditary lymphedema in human subjects.</p>		

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**SCREENING AND THERAPY FOR
LYMPHATIC DISORDERS INVOLVING THE
FLT4 RECEPTOR TYROSINE KINASE (VEGFR-3)**

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FIELD OF THE INVENTION

10 The present invention relates generally to the fields of molecular biology and medicine; more particularly to the areas of genetic screening and the identification and treatment of hereditary disorders; and more particularly to identification and treatment of hereditary lymphedema.

DESCRIPTION OF RELATED ART

15 The lymphatic system is a complex structure organized in parallel fashion to the circulatory system. In contrast to the circulatory system, which utilizes the heart to pump blood throughout the body, the lymphatic system pumps lymph fluid using the inherent contractility of the lymphatic vessels. The lymphatic vessels are not interconnected in the same manner as the blood vessels, but rather form a set of
20 coordinated structures including the initial lymphatic sinuses [Jeltsch *et al.*, (1997), *Science*, 276:1423-1425; and Castenholz, A., in Olszewski, W.L. (ed.), *Lymph Stasis: Pathophysiology, Diagnosis, and Treatment*. CRC Press: Boca Raton, Florida (1991), pp.15-42] which drain into the lymphatic capillaries and subsequently to the collecting lymphatics which drain into the lymphatic trunks and the thoracic duct which
25 ultimately drains into the venous circulation. The composition of the channels through which lymph passes is varied [Olszewski, W.L., in Olszewski, W.L. (ed.), *Lymph Stasis: Pathophysiology, Diagnosis, and Treatment*. CRC Press: Boca Raton, Florida (1991), pp. 235-258; and Kinmonth, J.B., in Kinmonth, J.B. (ed.), *The Lymphatics: Diseases, Lymphography and Surgery*. Edward Arnold Publishers: London, England
30 (1972), pp. 82-86], including the single epithelial layers of the initial lymphatics, the

multiple layers of the collecting lymphatics including endothelium, muscular and adventitial layers, and the complex organization of the lymph node. The various organs of the body such as skin, lung, and GI tract have components of the lymphatics with various unique features. [See Ohkuma, M., in Olszewski (1991), *supra*, at pp. 5 157-190; Uhley, H. and Leeds, S., in Olszewski (1991), *supra*, at pp. 191-210; and Barrowman, J.A., in Olszewski (1991), at pp. 221-234.]

Molecular biology has identified at least a few genes and proteins postulated to have roles mediating the growth and/or embryonic development of the lymphatic system. One such gene/protein is the receptor tyrosine kinase designated 10 Flt4 (fms-like tyrosine kinase 4), cloned from human erythroleukaemia cell and placental cDNA libraries [Aprelikova *et al.*, *Cancer Res.*, 52: 746-748 (1992); Galland *et al.*, *Genomics*, 13: 475-478 (1992); Galland *et al.*, *Oncogene*, 8: 1233-1240 (1993); and Pajusola *et al.*, *Cancer Res.*, 52:5738-5743 (1992)]. Studies showed that, in mouse embryos, a targeted disruption of the *Flt4* gene leads to a failure of the 15 remodeling of the primary vascular network, and death after embryonic day 9.5 [Dumont *et al.*, *Science*, 282: 946-949 (1998)]. These studies suggested that *Flt4* has an essential role in the development of the embryonic vasculature, before the emergence of the lymphatic vessels. However, additional studies indicated that, during further development, the expression of *Flt4* becomes restricted mainly to lymphatic 20 vessels [Kaipainen, A., *et al.*, *Proc. Natl. Acad. Sci. USA*, 92: 3566-3570 (1995)].

In humans, there are two isoforms of the Flt4 protein, designated as Flt4s (short, Genbank Accession No. X68203) and Flt4l (long, Genbank Accession Nos. X68203 and S66407, SEQ ID NO: 1). The sequence of these isoforms is largely identical, except for divergence that occurs at the carboxyl terminus of the receptor as 25 a result of alternative mRNA splicing at the 3' end. The C-terminus of the long form contains three tyrosyl residues, and one of them (Y1337 (SEQ ID NO: 2)) serves as an autophosphorylation site in the receptor [Fournier *et al.*, *Oncogene*, 11: 921-931 (1995); and Pajusola, *Oncogene*, 8: 2931-2937 (1993)]. Only the long form is detected in human erythroleukaemia (HEL) and in a megakaryoblastic cell line (the 30 DAMI cells), and the mouse *Flt4* gene (Genbank Accession No. L07296) only produces one mRNA transcript, corresponding to Flt4l [Galland *et al.*, *Oncogene*, 8: 1233-1240 (1993); and Pajusola *et al.*, *Cancer Res.*, 52: 5738-5743 (1992)]. These findings suggest that the long form of Flt4 may be responsible for most of the

biological properties of this receptor. The Flt4 protein is glycosylated and proteolytically processed in transfected cells [Pajusola *et al.*, *Oncogene*, 9: 3545-3555 (1994)]. During this process, the 175 kD form of the receptor matures to a 195 kD form, which is subsequently cleaved into a 125 kD C-terminal fragment, and a 75 kD extracellular domain-containing fragment, which are linked by disulphide bonding in the mature receptor.

Two growth factors, named vascular endothelial growth factors C and D (VEGF-C and VEGF-D) due to amino acid sequence similarity to earlier-discovered vascular endothelial growth factor, have been shown to bind and activate the tyrosine phosphorylation of Flt4. [Achen *et al.*, *Proc. Natl. Acad. Sci. USA*, 95: 548-553 (1998); and Joukov *et al.*, *EMBO J.*, 15: 290-298 (1996)]. Because of Flt4's growth factor binding properties and the fact that Flt4 possesses amino acid sequence similarity to two previously identified VEGF receptors (Flt1/VEGFR-1 and KDR/VEGFR-2), Flt4 has also been designated VEGFR-3, and these terms are used interchangeably herein.

When VEGF-C was intentionally overexpressed under a basal keratin promoter in transgenic mice, a hyperplastic lymphatic vessel network in the skin was observed. [Jeltsch *et al.*, *Science*, 276:1423-1425 (1997).] The results of this study, when combined with the expression pattern of VEGFR-3 in the lymphatic vasculature, suggest that lymphatic growth may be induced by VEGF-C and mediated via VEGFR-3. Notwithstanding the foregoing insights involving one cell surface receptor and the two apparent ligands therefor, little is known about the developmental regulation of the lymphatic system.

Hereditary or primary lymphedema, first described by Milroy in 1892 [Milroy, *N.Y. Med. J.*, 56:505-508 (1892)], is a developmental disorder of the lymphatic system which leads to a disabling and disfiguring swelling of the extremities. Hereditary lymphedema generally shows an autosomal dominant pattern of inheritance with reduced penetrance, variable expression, and variable age-at-onset [Greenlee *et al.*, *Lymphology*, 26:156-168 (1993)]. Swelling may appear in one or all limbs, varying in degree and distribution. If untreated, such swelling worsens over time. In rare instances, angiosarcoma may develop in affected tissues [Offori *et al.*, *Clin. Exp. Dermatol.*, 18:174-177 (1993)]. Despite having been described over a century ago, little progress has been made in understanding the mechanisms causing lymphedema.

5 A long-felt need exists for the identification of the presumed genetic variations that underlie hereditary lymphedema, to permit better informed genetic counseling in affected families, earlier diagnosis and treatment, and the development of more targeted and effective lymphedema therapeutic regimens. In addition, identification of genetic markers and high risk members of lymphedema families facilitates the identification and management of environmental factors that influence the expression and severity of a lymphedema phenotype.

SUMMARY OF THE INVENTION

10 The present invention provides materials and methods that address one or more of the long-felt needs identified above by identifying a genetic marker that correlates and is posited to have a causative role in the development of hereditary lymphedema. The invention is based in part on the discovery that, in several families with members afflicted with hereditary lymphedema, the lymphedema phenotype correlates with genetic markers localized to chromosome 5q34-q35; and that in at least
15 one such family, a missense mutation in the VEGFR-3 gene (which maps to chromosome 5q34-q35) exists that appears to behave in a dominant negative manner to interrupt tyrosine kinase signaling of the receptor. In view of the fact that VEGFR-3 acts as a high affinity receptor for vascular endothelial growth factor C (VEGF-C), a growth factor whose effects include modulation of the growth of the lymphatic
20 vascular network, these linkage and biochemical studies provide an important marker for determining a genetic predisposition for lymphedema in healthy individuals; and for diagnosing hereditary lymphedema in symptomatic individuals. Materials and methods for performing such genetic analyses are considered aspects of the present invention.

25 Thus, the invention provides genetic screening procedures that entail analyzing a person's genome -- in particular their *VEGFR-3* alleles -- to determine whether the individual possesses a genetic characteristic found in other individuals that are considered to be afflicted with, or at risk for, developing hereditary lymphedema.

30 For example, in one embodiment, the invention provides a method for determining a hereditary lymphedema development potential in a human subject comprising the steps of analyzing the coding sequence of the VEGFR-3 genes from the human subject; and determining hereditary lymphedema development potential in said human subject from the analyzing step.

In another embodiment, the invention provides a method of screening a human subject for an increased risk of developing a lymphatic disorder, comprising the steps of: (a) assaying nucleic acid of a human subject to determine a presence or an absence of a mutation altering the encoded VEGFR-3 amino acid sequence or
5 expression of at least one VEGFR-3 allele; and (b) screening for an increased risk of developing a lymphatic disorder from the presence or absence of said mutation.

By "human subject" is meant any human being, human embryo, or human fetus. It will be apparent that methods of the present invention will be of particular interest to individuals that have themselves been diagnosed with lymphedema
10 or have relatives that have been diagnosed with lymphedema.

By "screening for an increased risk" is meant determination of whether a genetic variation exists in the human subject that correlates with a greater likelihood of developing lymphedema than exists for the human population as a whole, or for a relevant racial or ethnic human sub-population to which the individual belongs. Both
15 positive and negative determinations (i.e., determinations that a genetic predisposition marker is present or is absent) are intended to fall within the scope of screening methods of the invention. In preferred embodiments, the presence of a mutation altering the sequence or expression of at least one *Flt4* receptor tyrosine kinase allele in the nucleic acid is correlated with an increased risk of developing a lymphatic
20 disorder, whereas the absence of such a mutation is reported as a negative determination.

By "lymphatic disorder" is meant any clinical condition affecting the lymphatic system, including but not limited to lymphedemas, lymphangiomas, lymphangiosarcomas, lymphangiomatosis, lymphangiectasis, and cystic hygroma.
25 Preferred embodiments are methods of screening a human subject for an increased risk of developing a lymphedema disorder, i.e., any disorder that physicians would diagnose as lymphedema and that is characterized by swelling associated with lymph accumulation, other than lymphedemas for which non-genetic causes (e.g., parasites, surgery) are known. By way of example, lymphedema disorders include Milroy-Nonne
30 (OMIM 153100) syndrome-early onset lymphedema [Milroy, *N.Y. Med. J.*, 56:505-508 (1892); and Dale, *J. Med. Genet.*, 22: 274-278 (1985)] and lymphedema praecox (Meige syndrome, OMIM 153200)-late onset lymphedema [Lewis *et al.*, *J. Ped.*, 104:641-648 (1984); Holmes *et al.*, *Pediatrics* 61:575-579 (1978); and Wheeler *et al.*,

Plastic Reconstructive Surg., 67:362-364 (1981)] which generally are described as separate entities, both characterized by dominant inheritance. However, there is confusion in the literature about the separation of these disorders. In Milroy's syndrome, the presence of edema, which is usually more severe in the lower
5 extremities, is seen from birth. Lymphedema praecox presents in a similar fashion but the onset of swelling is usually around puberty. Some cases have been reported to develop in the post-pubertal period. In the particular analyses described herein, the lymphedema families showing linkage to 5q34-q35 show an early onset for most affected individuals, but individuals in these pedigrees have presented during or after
10 puberty.

The "assaying" step of the invention may involve any techniques available for analyzing nucleic acid to determine its characteristics, including but not limited to well-known techniques such as single-strand conformation polymorphism analysis (SSCP) [Orita *et al.*, *Proc Natl. Acad. Sci. USA*, 86: 2766-2770 (1989)];
15 heteroduplex analysis [White *et al.*, *Genomics*, 12: 301-306 (1992)]; denaturing gradient gel electrophoresis analysis [Fischer *et al.*, *Proc. Natl. Acad. Sci. USA*, 80: 1579-1583 (1983); and Riesner *et al.*, *Electrophoresis*, 10: 377-389 (1989)]; DNA sequencing; RNase cleavage [Myers *et al.*, *Science*, 230: 1242-1246 (1985)]; chemical cleavage of mismatch techniques [Rowley *et al.*, *Genomics*, 30: 574-582 (1995); and
20 Roberts *et al.*, *Nucl. Acids Res.*, 25: 3377-3378 (1997)]; restriction fragment length polymorphism analysis; single nucleotide primer extension analysis [Shumaker *et al.*, *Hum. Mutat.*, 7: 346-354 (1996); and Pastinen *et al.*, *Genome Res.*, 7: 606-614 (1997)]; 5' nuclease assays [Pease *et al.*, *Proc. Natl. Acad. Sci. USA*, 91:5022-5026 (1994)]; DNA Microchip analysis [Ramsay, G., *Nature Biotechnology*, 16: 40-48
25 (1999); and Chee *et al.*, U.S. Patent No. 5,837,832]; and ligase chain reaction [Whiteley *et al.*, U.S. Patent No. 5,521,065]. [See generally, Schafer and Hawkins, *Nature Biotechnology*, 16: 33-39 (1998).] All of the foregoing documents are hereby incorporated by reference in their entirety.

In one preferred embodiment, the assaying involves sequencing of
30 nucleic acid to determine nucleotide sequence thereof, using any available sequencing technique. [See, *e.g.*, Sanger *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 74: 5463-5467 (1977) (dideoxy chain termination method); Mirzabekov, *TIBTECH*, 12: 27-32 (1994) (sequencing by hybridization); Drmanac *et al.*, *Nature Biotechnology*, 16: 54-58

(1998); U.S. Patent No. 5,202,231; and *Science*, 260: 1649-1652 (1993) (sequencing by hybridization); Kieleczawa *et al.*, *Science*, 258: 1787-1791 (1992) (sequencing by primer walking); (Douglas *et al.*, *Biotechniques*, 14: 824-828 (1993) (Direct sequencing of PCR products); and Akane *et al.*, *Biotechniques* 16: 238-241 (1994);
5 Maxam and Gilbert, *Meth. Enzymol.*, 65: 499-560 (1977) (chemical termination sequencing), all incorporated herein by reference.] The analysis may entail sequencing of the entire *VEGFR-3* gene genomic DNA sequence, or portions thereof; or sequencing of the entire *VEGFR-3* coding sequence or portions thereof. In some circumstances, the analysis may involve a determination of whether an individual
10 possesses a particular *VEGFR-3* allelic variant, in which case sequencing of only a small portion of nucleic acid -- enough to determine the sequence of a particular codon characterizing the allelic variant -- is sufficient. This approach is appropriate, for example, when assaying to determine whether one family member inherited the same allelic variant that has been previously characterized for another family member, or,
15 more generally, whether a person's genome contains an allelic variant that has been previously characterized and correlated with heritable lymphedema.

In another embodiment, the assaying step comprises performing a hybridization assay to determine whether nucleic acid from the human subject has a nucleotide sequence identical to or different from one or more reference sequences. In
20 a preferred embodiment, the hybridization involves a determination of whether nucleic acid derived from the human subject will hybridize with one or more oligonucleotides, wherein the oligonucleotides have nucleotide sequences that correspond identically to a portion of the *VEGFR-3* gene sequence, preferably the *VEGFR-3* coding sequence set forth in SEQ ID NO: 1, or that correspond identically except for one mismatch.
25 The hybridization conditions are selected to differentiate between perfect sequence complementarity and imperfect matches differing by one or more bases. Such hybridization experiments thereby can provide single nucleotide polymorphism sequence information about the nucleic acid from the human subject, by virtue of knowing the sequences of the oligonucleotides used in the experiments.

30 Several of the techniques outlined above involve an analysis wherein one performs a polynucleotide migration assay, *e.g.*, on a polyacrylamide electrophoresis gel, under denaturing or non-denaturing conditions. Nucleic acid derived from the human subject is subjected to gel electrophoresis, usually adjacent to

one or more reference nucleic acids, such as reference VEGFR-3 sequences having a coding sequence identical to all or a portion of SEQ ID NO: 1, or identical except for one known polymorphism. The nucleic acid from the human subject and the reference sequence(s) are subjected to similar chemical or enzymatic treatments and then
5 electrophoresed under conditions whereby the polynucleotides will show a differential migration pattern, unless they contain identical sequences. [See generally Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, New York: John Wiley & Sons, Inc. (1987-1999); and Sambrook *et al.*, (eds.), *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press
10 (1989), both incorporated herein by reference in their entirety.]

In the context of assaying, the term "nucleic acid of a human subject" is intended to include nucleic acid obtained directly from the human subject (*e.g.*, DNA or RNA obtained from a biological sample such as a blood, tissue, or other cell or fluid sample); and also nucleic acid derived from nucleic acid obtained directly from the
15 human subject. By way of non-limiting examples, well known procedures exist for creating cDNA that is complementary to RNA derived from a biological sample from a human subject, and for amplifying (*e.g.*, via polymerase chain reaction (PCR)) DNA or RNA derived from a biological sample obtained from a human subject. Any such derived polynucleotide which retains relevant nucleotide sequence information of the
20 human subject's own DNA/RNA is intended to fall within the definition of "nucleic acid of a human subject" for the purposes of the present invention.

In the context of assaying, the term "mutation" includes addition, deletion, and/or substitution of one or more nucleotides in the *VEGFR-3* gene sequence. The invention is demonstrated by way of non-limiting examples set forth
25 below that identify several mutations in *VEGFR-3*, including single nucleotide polymorphisms that introduce missense mutations into the *VEGFR-3* coding sequence (as compared to the *VEGFR-3* cDNA sequence set forth in SEQ ID NO: 1) and other polymorphisms that occur in introns and that are identifiable via sequencing, restriction fragment length polymorphism, or other techniques. Example 2 provides an assay to
30 determine whether a *VEGFR-3* mutation inhibits VEGFR-3 signaling. Additional assays to study both ligand binding and signaling activities of VEGFR-3 are disclosed, *e.g.*, in U.S. Patent No. 5,776,755 and International Patent Publication No. WO 98/33917, published 06 August 1998, both of which are incorporated herein by

reference in their entirety. Evidence that a VEGFR-3 mutation inhibits VEGFR-3 signaling is evidence that the mutation may have a causative role in lymphedema phenotype. However, even mutations that have no apparent causative role may serve as useful markers for heritable lymphedema, provided that the appearance of the mutation correlates reliably with the appearance of lymphedema.

In a related embodiment, the invention provides a method of screening for a VEGFR-3 hereditary lymphedema genotype in a human subject, comprising the steps of: (a) providing a biological sample comprising nucleic acid from a human subject; (b) analyzing the nucleic acid for the presence of a mutation or mutations in a VEGFR-3 allele in the nucleic acid of the human subject; (c) determining a VEGFR-3 genotype from said analyzing step; and (d) correlating the presence of a mutation in a VEGFR-3 allele with a hereditary lymphedema genotype. In a preferred embodiment, the biological sample is a cell sample containing human cells that contain genomic DNA of the human subject.

Although more time consuming and expensive than methods involving nucleic acid analysis, the invention also may be practiced by assaying protein of a human subject to determine the presence or absence of an amino acid sequence variation in VEGFR-3 protein from the human subject. Such protein analyses may be performed, e.g., by fragmenting VEGFR-3 protein via chemical or enzymatic methods and sequencing the resultant peptides; or by Western analyses using an antibody having specificity for a particular allelic variant of VEGFR-3.

The invention also provides materials that are useful for performing methods of the invention. For example, the present invention provides oligonucleotides useful as probes in the many analyzing techniques described above. In general, such oligonucleotide probes comprise 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides that have a sequence that is identical, or exactly complementary, to a portion of a human VEGFR-3 gene sequence, or that is identical or exactly complementary except for one nucleotide substitution. In a preferred embodiment, the oligonucleotides have a sequence that corresponds in the foregoing manner to a human VEGFR-3 coding sequence, and in particular, the VEGFR-3 coding sequence set forth in SEQ ID NO: 1. In one variation, an oligonucleotide probe of the invention is purified and isolated. In another

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variation, the oligonucleotide probe is labeled, *e.g.*, with a radioisotope, chromophore, or fluorophore. In yet another variation, the probe is covalently attached to a solid support. [See generally Ausubel *et al.* And Sambrook *et al.*, *supra*.]

In a particularly preferred embodiment, the invention comprises an
5 oligonucleotide probe useful for detecting the P1114L mutation (missense mutation at nucleotide 3360 of SEQ ID NO: 1, causing a proline to leucine change at residue 1114 in SEQ ID NO: 2) that has been characterized herein in affected individuals of one lymphedema family. For example, the invention provides oligonucleotides comprising anywhere from 6 to 50 nucleotides that have a sequence that is identical to, or exactly
10 complementary to, a portion of the human VEGFR-3 coding sequence set forth in SEQ ID NO: 1, except for a nucleotide substitution corresponding to nucleotide 3360 of SEQ ID NO: 1. Such oligonucleotides may be generically described by the formula X_nYZ_m or its complement; where n and m are integers from 0 to 49; where $5 \leq (n + m) \leq 49$; where X_n is a stretch of n nucleotides identical to a first portion of SEQ ID NO:
15 1 and Z_m is a stretch of m nucleotides identical to a second portion of SEQ ID NO: 1, wherein the first and second portions are separated in SEQ ID NO: 1 by one nucleotide; and wherein Y represents a nucleotide other than the nucleotide that separates the first and second portions of SEQ ID NO: 1. For example, where X_n represents 0 to 49 nucleotides immediately upstream (5') of nucleotide 3360 of SEQ
20 ID NO: 1 and Z_m represents 0 to 49 nucleotides immediately downstream (3') of nucleotide 3360 of SEQ ID NO: 1, Y represents a nucleotide other than cytosine, since a cytosine nucleotide is found at position 3360 of SEQ ID NO: 1. In a preferred embodiment, Y is a thymine nucleotide.

In a related embodiment, the invention provides a kit comprising at
25 least two such oligonucleotide probes. Preferably, the two or more probes are provided in separate containers, or attached to separate solid supports, or attached separately to the same solid support, *e.g.*, on a DNA microchip.

In still another related embodiment, the invention provides an array of oligonucleotide probes immobilized on a solid support, the array having at least 4
30 probes, preferably at least 100 probes, and preferably up to 100,000, 10,000, or 1000 probes, wherein each probe occupies a separate known site in the array. In a preferred embodiment, the array includes probe sets comprising two to four probes, wherein one probe is exactly identical or exactly complementary to a human VEGFR-3 coding

sequence, and the other one to three members of the set are exactly identical to the first member, but for at least one different nucleotide, which different nucleotide is located in the same position in each of the one to three additional set members. In one preferred embodiment, the array comprises several such sets of probes, wherein the sets correspond to different segments of the human VEGFR-3 gene sequence. In a highly preferred embodiment, the array comprises enough sets of oligonucleotides of length N to correspond to every particular N-mer sequence of the VEGFR-3 gene, where N is preferably 6 to 25 and more preferably 9 to 20. Materials and methods for making such probes are known in the art and are described, for example, in U.S. Patent Nos. 5,837,832, 5,202,231, 5,002,867, and 5,143,854.

Moreover, the discoveries which underlie the present invention identify a target for therapeutic intervention in cases of hereditary lymphedema. The causative mutation in the family that has been studied in greatest detail is a mutation that appears to result in VEGFR-3 signaling that is reduced in heterozygous affected individuals, but not completely eliminated. This data supports a therapeutic indication for administration of agents, such as VEGFR-3 ligand polypeptides, that will induce VEGFR-3 signaling in the lymphatic endothelia of affected individuals to effect improvement in the structure and function of the lymphatic vasculature of such individuals. In addition, therapeutic gene therapy, to replace defective VEGFR-3 alleles or increase production of VEGFR-3 ligand polypeptides *in vivo*, is envisioned as an aspect of the invention.

Thus, in yet another aspect, the invention provides a therapeutic or prophylactic method of treatment for lymphedema, comprising the step of administering to a mammalian subject in need of therapeutic or prophylactic treatment for lymphedema a composition comprising a compound effective to induce intracellular signaling of VEGFR-3 in lymphatic endothelial cells that express said receptor. In a preferred embodiment, the compound comprises a polypeptide ligand for VEGFR-3, or a polynucleotide encoding such a ligand, wherein the polynucleotide is administered in a form that results in transcription and translation of the polynucleotide in the mammalian subject to produce the ligand *in vivo*. In another preferred embodiment, the compound comprises any small molecule that is capable of binding to the VEGFR-3 receptor extracellular domain and inducing intracellular signaling.

For example, the invention provides a therapeutic or prophylactic method of treatment for lymphedema, comprising the step of administering to a mammalian subject in need of therapeutic or prophylactic treatment for lymphedema a composition comprising a polynucleotide, the polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor C (VEGF-C) polypeptide. In a preferred embodiment, the subject is a human subject.

While it is contemplated that the VEGF-C polynucleotide could be administered purely as a prophylactic treatment to prevent lymphedema in subjects at risk for developing lymphedema, it is contemplated in a preferred embodiment that the polynucleotide be administered to subjects afflicted with lymphedema, for the purpose of ameliorating its symptoms (e.g., swelling due to the accumulation of lymph). The polynucleotide is included in the composition in an amount and in a form effective to promote expression of a VEGF-C polypeptide in or near the lymphatic endothelia of the mammalian subject, to stimulate VEGFR-3 signaling in the lymphatic endothelia of the subject.

In a preferred embodiment, the mammalian subject is a human subject. Practice of methods of the invention in other mammalian subjects, especially mammals that are conventionally used as models for demonstrating therapeutic efficacy in humans (e.g., primate, porcine, canine, equine, murine, or rabbit animals), also is contemplated. Several potential animal models for hereditary lymphedema have been described in the literature. [See, e.g., Lyon *et al.*, *Mouse News Lett.* 71: 26 (1984), *Mouse News Lett.* 74: 96 (1986), and *Genetic variants and strains of the laboratory mouse*, 2nd ed., New York: Oxford University Press (1989), p. 70 (*Chylous ascites mouse*); Dumont *et al.*, *Science*, 282: 946-949 (1998) (heterozygous VEGFR-3 knockout mouse); Patterson *et al.*, "Hereditary Lymphedema," *Comparative Pathology Bulletin*, 3: 2 (1971) (canine hereditary lymphedema model); van der Putte, "Congenital Hereditary Lymphedema in the Pig," *Lympho*, 11: 1-9 (1978); and Campbell-Beggs *et al.*, "Chyloabdomen in a neonatal foal," *Veterinary Record*, 137: 96-98 (1995).] Those models which are determined to have analogous mutations to the VEGFR-3 gene are preferred. In another embodiment, "knock in" homologous recombination genetic engineering strategies are used to create an animal model (e.g., a mouse model) having a VEGFR-3 allelic variation analogous to the human variations described herein. [See, e.g., Partanen *et al.*, *Genes & Development*, 12: 2332-2344

(1998) (gene targeting to introduce mutations into a receptor protein (FGFR-1) in mice).] Such mice can also be bred to the heterozygous VEGFR-3 knockout mice or *Chy* mice described above to further modify the phenotypic severity of the lymphedema disease.

5 For the practice of methods of the invention, the term "VEGF-C polypeptide" is intended to include any polypeptide that has a VEGF-C or VEGF-C analog amino acid sequence (as defined elsewhere herein in greater detail) and that is able to bind the VEGFR-3 extracellular domain and stimulate VEGFR-3 signaling *in vivo*. The term "VEGF-C polynucleotide" is intended to include any polynucleotide
10 (*e.g.*, DNA or RNA, single- or double-stranded) comprising a nucleotide sequence that encodes a VEGF-C polypeptide. Due to the well-known degeneracy of the genetic code, multiple VEGF-C polynucleotide sequences exist that encode any selected VEGF-C polypeptide.

15 For treatment of humans, VEGF-C polypeptides with an amino acid sequence of a human VEGF-C are highly preferred, and polynucleotides comprising a nucleotide sequence of a human VEGF-C cDNA are highly preferred. By "human VEGF-C" is meant a polypeptide corresponding to a naturally occurring protein (prepro-protein, partially-processed protein, or fully-processed mature protein) encoded by any allele of the human VEGF-C gene, or a polypeptide comprising a
20 biologically active fragment of a naturally-occurring mature protein. By way of example, a human VEGF-C comprises a continuous portion of the amino acid sequence set forth in SEQ ID NO: 4 sufficient to permit the polypeptide to bind and stimulate VEGFR-3 phosphorylation in cells that express such receptors. A polypeptide comprising amino acids 131-211 of SEQ ID NO: 4 is specifically
25 contemplated. For example, polypeptides having an amino acid sequence comprising a continuous portion of SEQ ID NO: 4, the continuous portion having, as its amino terminus, an amino acid selected from the group consisting of positions 30-131 of SEQ ID NO: 4, and having, as its carboxyl terminus, an amino acid selected from the group consisting of positions 211-419 of SEQ ID NO: 4 are contemplated. An amino
30 terminus selected from the group consisting of positions 102-131 of SEQ ID NO: 4 is preferred, and an amino terminus selected from the group consisting of positions 103-113 of SEQ ID NO: 4 is highly preferred. Likewise, a carboxyl terminus selected from the group consisting of positions 211-227 of SEQ ID NO: 4 is preferred. As

stated above, the term "human VEGF-C" also is intended to encompass polypeptides encoded by allelic variants of the human VEGF-C characterized by the sequences set forth in SEQ ID NOs: 3 & 4.

Moreover, since the therapeutic VEGF-C is to be administered as
5 recombinant VEGF-C or indirectly via somatic gene therapy, it is within the skill in the art to make and use analogs of human VEGF-C (and polynucleotides that encode such analogs) wherein one or more amino acids have been added, deleted, or replaced with other amino acids, especially with conservative replacements, and wherein the VEGFR-3-stimulatory biological activity has been retained. Analogs that retain
10 VEGFR-3-stimulatory VEGF-C biological activity are contemplated as VEGF-C polypeptides for use in the present invention. In a preferred embodiment, analogs having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 such modifications and that retain VEGFR-3-stimulatory VEGF-C biological activity are contemplated as VEGF-C polypeptides for use in the present invention.
15 Polynucleotides encoding such analogs are generated using conventional PCR, site-directed mutagenesis, and chemical synthesis techniques.

Also contemplated as VEGF-C polypeptides are non-human mammalian or avian VEGF-C polypeptides and polynucleotides. By "mammalian VEGF-C" is meant a polypeptide corresponding to a naturally occurring protein (prepro-protein,
20 partially-processed protein, or fully-processed mature protein) encoded by any allele of a VEGF-C gene of any mammal, or a polypeptide comprising a biologically active fragment of a mature protein. The term "mammalian VEGF-C polypeptide" is intended to include analogs of mammalian VEGF-C's that possess the *in vivo* VEGFR-3-stimulatory effects of the mammalian VEGF-C.

25 Irrespective of which encoded VEGF-C polypeptide is chosen, any VEGF-C polynucleotide gene therapy pharmaceutical encoding it preferably comprises a nucleotide sequence encoding a secretory signal peptide fused in-frame with the VEGF-C polypeptide sequence. The secretory signal peptide directs secretion of the VEGF-C polypeptide by the cells that express the polynucleotide, and is cleaved by the
30 cell from the secreted VEGF-C polypeptide. For example, the VEGF-C polynucleotide could encode the complete prepro-VEGF-C sequence set forth in SEQ ID NO: 4; or could encode the VEGF-C signal peptide fused in-frame to a sequence encoding a fully-processed VEGF-C (e.g., amino acids 103-227 of SEQ ID NO: 4) or

VEGF-C analog. Moreover, there is no requirement that the signal peptide be derived from VEGF-C. The signal peptide sequence can be that of another secreted protein, or can be a completely synthetic signal sequence effective to direct secretion in cells of the mammalian subject.

5 In one embodiment, the VEGF-C polynucleotide of the invention comprises a nucleotide sequence that will hybridize to a polynucleotide that is complementary to the human VEGF-C cDNA sequence specified in SEQ ID NO: 3 under the following exemplary stringent hybridization conditions: hybridization at 42°C in 50% formamide, 5X SSC, 20 mM Na•PO₄, pH 6.8; and washing in 1X SSC at 10 55°C for 30 minutes; and wherein the nucleotide sequence encodes a polypeptide that binds and stimulates human VEGFR-3. It is understood that variation in these exemplary conditions occur based on the length and GC nucleotide content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining appropriate hybridization conditions. [See Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Second ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1989) §§ 9.47-9.51.] 15

In preferred embodiments, the VEGF-C polynucleotide further comprises additional sequences to facilitate the VEGF-C gene therapy. In one embodiment, a "naked" VEGF-C transgene (i.e., a transgene without a viral, liposomal, 20 or other vector to facilitate transfection) is employed for gene therapy. In this embodiment, the VEGF-C polynucleotide preferably comprises a suitable promoter and/or enhancer sequence (e.g., cytomegalovirus promoter/enhancer [Lehner *et al.*, *J. Clin. Microbiol.*, 29:2494-2502 (1991); Boshart *et al.*, *Cell*, 41:521-530 (1985)]; Rous sarcoma virus promoter [Davis *et al.*, *Hum. Gene Ther.*, 4:151 (1993)]; Tie 25 promoter [Korhonen *et al.*, *Blood*, 86(5): 1828-1835 (1995)]; or simian virus 40 promoter) for expression in the target mammalian cells, the promoter being operatively linked upstream (i.e., 5') of the VEGF-C coding sequence. The VEGF-C polynucleotide also preferably further includes a suitable polyadenylation sequence (e.g., the SV40 or human growth hormone gene polyadenylation sequence) operably 30 linked downstream (i.e., 3') of the VEGF-C coding sequence. The polynucleotide may further optionally comprise sequences whose only intended function is to facilitate large-scale production of the vector, e.g., in bacteria, such as a bacterial origin of replication and a sequence encoding a selectable marker. However, in a preferred

embodiment, such extraneous sequences are at least partially cleaved off prior to administration to humans according to methods of the invention. One can manufacture and administer such polynucleotides to achieve successful gene therapy using procedures that have been described in the literature for other transgenes. See, e.g.,
5 Isner *et al.*, *Circulation*, 91: 2687-2692 (1995); and Isner *et al.*, *Human Gene Therapy*, 7: 989-1011 (1996); incorporated herein by reference in the entirety.

Any suitable vector may be used to introduce the VEGF-C transgene into the host. Exemplary vectors that have been described in the literature include replication-deficient retroviral vectors, including but not limited to lentivirus vectors
10 [Kim *et al.*, *J. Virol.*, 72(1): 811-816 (1998); Kingsman & Johnson, *Scrip Magazine*, October, 1998, pp. 43-46.]; adeno-associated viral vectors [Gnatenko *et al.*, *J. Investig. Med.*, 45: 87-98 (1997)]; adenoviral vectors [See, e.g., U.S. Patent No. 5,792,453; Quantin *et al.*, *Proc. Natl. Acad. Sci. USA*, 89: 2581-2584 (1992); Stratford-Perricadet *et al.*, *J. Clin. Invest.*, 90: 626-630 (1992); and Rosenfeld *et al.*,
15 *Cell*, 68: 143-155 (1992)]; Lipofectin-mediated gene transfer (BRL); liposomal vectors [See, e.g., U.S. Patent No. 5,631,237 (Liposomes comprising Sendai virus proteins)] ; and combinations thereof. All of the foregoing documents are incorporated herein by reference in the entirety. Replication-deficient adenoviral vectors constitute a preferred embodiment.

20 In embodiments employing a viral vector, preferred polynucleotides still include a suitable promoter and polyadenylation sequence as described above. Moreover, it will be readily apparent that, in these embodiments, the polynucleotide further includes vector polynucleotide sequences (e.g., adenoviral polynucleotide sequences) operably connected to the sequence encoding a VEGF-C polypeptide.

25 Thus, in one embodiment the composition to be administered comprises a vector, wherein the vector comprises the VEGF-C polynucleotide. In a preferred embodiment, the vector is an adenovirus vector. In a highly preferred embodiment, the adenovirus vector is replication-deficient, i.e., it cannot replicate in the mammalian subject due to deletion of essential viral-replication sequences from the adenoviral
30 genome. For example, the inventors contemplate a method wherein the vector comprises a replication-deficient adenovirus, the adenovirus comprising the VEGF-C polynucleotide operably connected to a promoter and flanked on either end by adenoviral polynucleotide sequences.

The composition to be administered according to methods of the invention preferably comprises (in addition to the polynucleotide or vector) a pharmaceutically-acceptable carrier solution such as water, saline, phosphate-buffered saline, glucose, or other carriers conventionally used to deliver therapeutics intravascularly. Multi-gene therapy is also contemplated, in which case the composition optionally comprises both the VEGF-C polynucleotide/vector and another polynucleotide/vector. As described in greater detail below, a VEGF-D transgene is a preferred candidate for co-administration with the VEGF-C transgene.

The "administering" that is performed according to the present method may be performed using any medically-accepted means for introducing a therapeutic directly or indirectly into a mammalian subject to reach the lymph or the lymphatic system, including but not limited to injections; oral ingestion; intranasal or topical administration; and the like. In a preferred embodiment, administration of the composition comprising the VEGF-C polynucleotide is performed intravascularly, such as by intravenous or intra-arterial injection, or by subcutaneous injection or local depot administration. In a highly preferred embodiment, the composition is administered locally, *e.g.*, to the site of swelling.

In still another variation, endothelial cells or endothelial progenitor cells are transfected *ex vivo* with a wild type VEGFR-3 transgene, and the transfected cells are administered to the mammalian subject.

In another aspect, the invention provides a therapeutic or prophylactic method of treating for lymphedema, comprising the step of administering to a mammalian subject in need of treatment for lymphedema a composition comprising a VEGF-C polypeptide, in an amount effective to treat or prevent swelling associated with lymphedema. Administration via one or more intravenous or subcutaneous injections is contemplated. Co-administration of VEGF-C polynucleotides and VEGF-C polypeptides is also contemplated.

In yet another embodiment, the invention provides the use of a VEGF-C polynucleotide or VEGF-C polypeptide for the manufacture of a medicament for the treatment or prevention of lymphedema.

In still another embodiment, the invention provides a therapeutic or prophylactic method of treatment for lymphedema, comprising the step of administering to a mammalian subject in need of therapeutic or prophylactic treatment

of lymphedema a composition comprising a polynucleotide, the polynucleotide comprising a nucleotide sequence that encodes a vascular endothelial growth factor D (VEGF-D) polypeptide. Such methods are practiced essentially as described herein with respect to VEGF-C-encoding polynucleotides, except that polynucleotides
5 encoding VEGF-D are employed. A detailed description of the human VEGF-D gene and protein are provided in Achen, *et al.*, *Proc. Nat'l Acad. Sci. U.S.A.*, 95(2): 548-553 (1998); International Patent Publication No. WO 98/07832, published 26 February 1998; and in Genbank Accession No. AJ000185, all incorporated herein by reference. A cDNA and deduced amino acid sequence for prepro-VEGF-D is set forth herein in
10 SEQ ID NOs: 5 and 6. Of course, due to the well-known degeneracy of the genetic code, multiple VEGF-D encoding polynucleotide sequence exist, any of which may be employed according to the methods taught herein.

As described herein in detail with respect to VEGF-C, the use of polynucleotides that encode VEGF-D fragments, VEGF-D analogs, VEGF-D allelic
15 and interspecies variants, and the like which possess *in vivo* stimulatory effects of human VEGF-D are all contemplated as being encompassed by the present invention.

In yet another embodiment, the invention provides a therapeutic or prophylactic method of treatment for lymphedema, comprising the step of administering to a mammalian subject in need of treatment for lymphedema a
20 composition comprising a VEGF-C polypeptide, in an amount effective to treat or prevent swelling associated with lymphedema. Administration via one or more intravenous or subcutaneous injections is contemplated.

The VEGFR-3 allelic variant polynucleotides and polypeptides described herein that were discovered and characterized by the present inventors are
25 themselves considered aspects of the invention. Such polynucleotides and polypeptides are useful, for example, in screening assays to study the biological activities of VEGFR-3 variant alleles and identify compounds that are capable of modulating that activity, *e.g.*, to identify therapeutic candidates for treatment of lymphedema. Such screening assays are also considered aspects of the invention.

30 The polypeptides of the invention are intended to include complete VEGFR-3 polypeptides with signal peptide (*e.g.*, approximately residues 1 to 20 of SEQ ID NO: 2), mature VEGFR-3 polypeptides lacking any signal peptide, and recombinant variants wherein a foreign or synthetic signal peptide has been fused to

the mature VEGFR-3 polypeptide. Polynucleotides of the invention include all polynucleotides that encode all such polypeptides. It will be understood that for essentially any polypeptide, many polynucleotides can be constructed that encode the polypeptide by virtue of the well known degeneracy of the genetic code. All such polynucleotides are intended as aspects of the invention.

Thus, in yet another aspect, the invention provides a purified polynucleotide comprising a nucleotide sequence encoding a human VEGFR-3 protein variant, wherein said polynucleotide is capable of hybridizing to the complement of SEQ ID NO: 1 under stringent hybridization conditions, and wherein the encoded VEGFR-3 protein variant has an amino acid sequence that differs at position 1114 from the amino acid sequence set forth in SEQ ID NO: 1. Exemplary conditions are as follows: hybridization at 42°C in 50% formamide, 5X SSC, 20 mM Na₂PO₄, pH 6.8; and washing in 0.2X SSC at 55°C. It is understood by those of skill in the art that variation in these conditions occurs based on the length and GC nucleotide content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining appropriate hybridization conditions. [See Sambrook et al. (1989), *supra*, §§ 9.47-9.51.]

In a related embodiment, the invention provides a purified polynucleotide comprising a nucleotide sequence encoding a VEGFR-3 protein of a human that is affected with heritable lymphedema or other lymphatic disorder; wherein the polynucleotide is capable of hybridizing to the complement of SEQ ID NO: 1 under stringent hybridization conditions, and wherein the encoded polynucleotide has an amino acid sequence that differs from SEQ ID NO: 1 at at least one codon. It will be understood that conventional recombinant techniques can be used to isolate such polynucleotides from individuals affected with heritable lymphedema or their relatives. The wildtype VEGFR-3 cDNA sequence set forth in SEQ ID NO: 1 (or its complement, or fragments thereof) is used as a probe to identify and isolate VEGFR-3 sequences from nucleic acid derived from the individuals. Alternatively, PCR amplification primers based on the wildtype VEGFR-3 sequence are generated and used to amplify either VEGFR-3 genomic DNA or VEGFR-3 mRNA from the human subject. The resultant amplified genomic DNA or cDNA is sequenced to determine the variations that characterize the VEGFR-3 lymphedema allele of the individual. A preferred VEGFR-3 lymphedema allele is the P1114L allele described in detail herein.

In addition, the invention provides vectors that comprise the polynucleotides of the invention. Such vectors are useful for amplifying and expressing the VEGFR-3 proteins encoded by the polynucleotides. The invention further provides a host cell transformed or transfected with polynucleotides (including
5 vectors) of the invention. In a preferred embodiment, the host cell expresses the encoded VEGFR-3 protein on its surface. Such host cells are useful in cell-based screening assays for identifying modulators that stimulate or inhibit signaling of the encoded VEGFR-3. Modulators that stimulate VEGFR-3 signaling have utility as therapeutics to treat lymphedemas, whereas modulators that are inhibitory have utility
10 for treating hyperplastic lymphatic conditions mediated by the allelic variant VEGFR-3. In a preferred embodiment, host cells of the invention are co-transfected with both a wildtype and an allelic variant VEGFR-3 polynucleotide, such that the cells express both receptor types on their surface. Such host cells are preferred for simulating a heterozygous VEGFR-3 genotype of many individuals affected with lymphedema.

15 In yet another aspect, the invention provides a transgenic mouse characterized by a non-native VEGFR-3 allele that has been introduced into the mouse, and the transgenic progeny thereof. Preferred allelic variants include allelic variants that correlate with hereditary lymphedema in human subjects, such as an allelic variant wherein a P1114L missense mutation has been introduced into the murine
20 VEGFR-3 gene, or wherein the human P1114L allelic variant has been substituted for a murine VEGFR-3 allele. Such mice are produced using standard methods. [See, e.g., Hogan *et al.* (eds.), *Manipulating the Mouse Embryo*, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1986).]

25 In yet another aspect, the invention provides assays for identifying modulators of VEGFR-3 signaling, particularly modulators of the signaling of allelic variants of VEGFR-3 that correlate with lymphatic disorders such as heritable lymphedema. For example, the invention provides a method for identifying a modulator of intracellular VEGFR-3 signaling, comprising the steps of: contacting a cell expressing at least one mutant mammalian VEGFR-3 polypeptide in the presence
30 and in the absence of a putative modulator compound; b) detecting VEGFR-3 signaling in the cell; and c) identifying a putative modulator compound in view of decreased or increased signaling in the presence of the putative modulator, as compared to signaling in the absence of the putative modulator.

By "mutant mammalian VEGFR-3 polypeptide" is meant a VEGFR-3 polypeptide that varies from a wildtype mammalian VEGFR-3 polypeptide (e.g., by virtue of one or more amino acid additions, deletions, or substitutions), wherein the variation is reflective of a naturally occurring variation that has been correlated with a lymphatic disorder, such as lymphedema. By way of example, a P1114L substitution variation at position 1114 (SEQ ID NO: 1) of human VEGFR-3 has been correlated with heritable lymphedema. The P1114L human allelic variant, or an analogous human allelic variant having a different substitution at position 1114, or a non-human VEGFR-3 into which a mutation at the position corresponding to P1114 has been introduced are all examples of mutant mammalian VEGFR-3 polypeptides.

The detecting step can entail the detection of any parameter indicative of VEGFR-3 signaling. For example, the detecting step can entail a measurement of VEGFR-3 autophosphorylation, or a measurement of VEGFR-3-mediated cell growth, or a measurement of any step in the VEGFR-3 signaling cascade between VEGFR-3 autophosphorylation and cell growth.

In a preferred embodiment, the method is practiced with a cell that expresses the mutant mammalian VEGFR-3 polypeptide and a wildtype mammalian VEGFR-3 polypeptide. Such cells are thought to better mimic the conditions in heterozygous individuals suffering from a VEGFR-3-mediated lymphatic disorder. In a highly preferred embodiment, the mutant and wildtype VEGFR-3 polypeptides are human. In a very highly preferred embodiment, the mutant VEGFR-3 polypeptide comprises a leucine amino acid at the position corresponding to position 1114 of SEQ ID NO: 1.

Additional features and variations of the invention will be apparent to those skilled in the art from the entirety of this application, including the drawing and detailed description, and all such features are intended as aspects of the invention. Likewise, features of the invention described herein can be re-combined into additional embodiments that are also intended as aspects of the invention, irrespective of whether the combination of features is specifically mentioned above as an aspect or embodiment of the invention. Also, only such limitations which are described herein as critical to the invention should be viewed as such; variations of the invention lacking limitations which have not been described herein as critical are intended as aspects of the invention.

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In addition to the foregoing, the invention includes, as an additional aspect, all embodiments of the invention narrower in scope in any way than the variations specifically mentioned above. Although the applicant(s) invented the full scope of the claims appended hereto, the claims appended hereto are not intended to encompass within their scope the prior art work of others. Therefore, in the event that statutory prior art within the scope of a claim is brought to the attention of the applicants by a Patent Office or other entity or individual, the applicant(s) reserve the right to exercise amendment rights under applicable patent laws to redefine the subject matter of such a claim to specifically exclude such statutory prior art or obvious variations of statutory prior art from the scope of such a claim. Variations of the invention defined by such amended claims also are intended as aspects of the invention.

BRIEF DESCRIPTION OF THE DRAWING

Figures 1A-1F depict pedigrees of six hereditary lymphedema families (Families 101, 106, 111, 135, 105, and 127, respectively) informative for linkage. Filled symbols represent individuals with clinically documented lymphedema. Crossed symbols represent individuals with an ambiguous phenotype. An ambiguous phenotype is defined as self-reported swelling of the limbs with no known cause, without a clinical diagnosis of lymphedema. Individuals of ambiguous phenotype were coded as disease status unknown for the linkage analysis. The proband in each family is indicated by an arrow.

Figure 2 is a graph summarizing VITESSE analysis of lymphedema families with markers localized to chromosome 5q34-q35. In the graph, filled circles represent analyses for Families 101, 105, 106, and 111; open boxes represent analyses for Families 101, 106, and 111; open circles represent the VEGFR-3 gene; and open triangles represent Family 135. The one LOD confidence interval lies completely within the interval flanked by markers D5S1353 and D5S408 and overlaps the most likely location of *Flt4* (*VEGFR-3*). Linkage is excluded for the entire region for family 135.

DETAILED DESCRIPTION OF THE INVENTION

Certain therapeutic aspects of the present invention involve the administration of Vascular Endothelial Growth Factor C or D polynucleotides and

polypeptides. The growth factor VEGF-C, as well as native human, non-human mammalian, and avian polynucleotide sequences encoding VEGF-C, and VEGF-C variants and analogs, have been described in detail in International Patent Application Number PCT/US98/01973, filed 02 February 1998 and published on 06 August 1998 as International Publication Number WO 98/33917; in Joukov *et al.*, *J. Biol. Chem.*, 273(12): 6599-6602 (1998); and in Joukov *et al.*, *EMBO J.*, 16(13): 3898-3911 (1997), all of which are incorporated herein by reference in the entirety. As explained therein in detail, human VEGF-C is initially produced in human cells as a prepro-VEGF-C polypeptide of 419 amino acids. A cDNA and deduced amino acid sequence for human prepro-VEGF-C are set forth in SEQ ID NOs: 3 and 4, respectively, and a cDNA encoding human VEGF-C has been deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209 (USA), pursuant to the provisions of the Budapest Treaty (Deposit date of 24 July 1995 and ATCC Accession Number 97231). VEGF-C sequences from other species have also been reported. See Genbank Accession Nos. MMU73620 (*Mus musculus*); and CCY15837 (*Coturnix coturnix*) for example, incorporated herein by reference.

The prepro-VEGF-C polypeptide is processed in multiple stages to produce a mature and most active VEGF-C polypeptide of about 21-23 kD (as assessed by SDS-PAGE under reducing conditions). Such processing includes cleavage of a signal peptide (SEQ ID NO: 4, residues 1-31); cleavage of a carboxyl-terminal peptide (corresponding approximately to amino acids 228-419 of SEQ ID NO: 4 and having a pattern of spaced cysteine residues reminiscent of a Balbiani ring 3 protein (BR3P) sequence [Dignam *et al.*, *Gene*, 88:133-40 (1990); Paulsson *et al.*, *J. Mol. Biol.*, 211:331-49 (1990)]) to produce a partially-processed form of about 29 kD; and cleavage (apparently extracellularly) of an amino-terminal peptide (corresponding approximately to amino acids 32-103 of SEQ ID NO: 4) to produce a fully-processed mature form of about 21-23 kD. Experimental evidence demonstrates that partially-processed forms of VEGF-C (*e.g.*, the 29 kD form) are able to bind the VEGFR-3 receptor, whereas high affinity binding to VEGFR-2 occurs only with the fully processed forms of VEGF-C.

Moreover, it has been demonstrated that amino acids 103-227 of SEQ ID NO: 4 are not all critical for maintaining VEGF-C functions. A polypeptide consisting of amino acids 113-213 (and lacking residues 103-112 and 214-227) of

SEQ ID NO: 2 retains the ability to bind and stimulate VEGFR-3, and it is expected that a polypeptide spanning from about residue 131 to about residue 211 will retain VEGF-C biological activity. The cysteine residue at position 156 has been shown to be important for VEGFR-2 binding ability. However, VEGF-C ΔC_{156} polypeptides
5 (i.e., analogs that lack this cysteine due to deletion or substitution) remain potent activators of VEGFR-3, and are therefore considered to be among the preferred candidates for treatment of lymphedema. The cysteine at position 165 of SEQ ID NO: 4 is essential for binding to either receptor, whereas analogs lacking the cysteines at positions 83 or 137 compete with native VEGF-C for binding with both receptors and
10 are able to stimulate both receptors.

An alignment of human VEGF-C with VEGF-C from other species (performed using any generally accepted alignment algorithm) suggests additional residues wherein modifications can be introduced (*e.g.*, insertions, substitutions, and/or deletions) without destroying VEGF-C biological activity. Any position at which
15 aligned VEGF-C polypeptides of two or more species have different amino acids, especially different amino acids with side chains of different chemical character, is a likely position susceptible to modification without concomitant elimination of function. An exemplary alignment of human, murine, and quail VEGF-C is set forth in Figure 5 of PCT/US98/01973.

20 Apart from the foregoing considerations, it will be understood that innumerable conservative amino acid substitutions can be performed to a wildtype VEGF-C sequence which are likely to result in a polypeptide that retains VEGF-C biological activities, especially if the number of such substitutions is small. By
25 "conservative amino acid substitution" is meant substitution of an amino acid with an amino acid having a side chain of a similar chemical character. Similar amino acids for making conservative substitutions include those having an acidic side chain (glutamic acid, aspartic acid); a basic side chain (arginine, lysine, histidine); a polar amide side chain (glutamine, asparagine); a hydrophobic, aliphatic side chain (leucine, isoleucine, valine, alanine, glycine); an aromatic side chain (phenylalanine, tryptophan, tyrosine); a
30 small side chain (glycine, alanine, serine, threonine, methionine); or an aliphatic hydroxyl side chain (serine, threonine). Addition or deletion of one or a few internal amino acids without destroying VEGF-C biological activities also is contemplated.

Without intending to be limited to a particular theory, the mechanism behind the efficacy of VEGF-C in treating or preventing lymphedema is believed to relate to the ability of VEGF-C to stimulate VEGFR-3 signaling. Administration of VEGF-C in quantities exceeding those usually found in interstitial fluids is expected to stimulate VEGFR-3 in human subjects who, by virtue of a dominant negative heterozygous mutation, have insufficient VEGFR-3 signaling.

The growth factor named Vascular Endothelial Growth Factor D (VEGF-D), as well as human sequences encoding VEGF-D, and VEGF-D variants and analogs, have been described in detail in International Patent Application Number PCT/US97/14696, filed 21 August 1997 and published on 26 February 1998 as International Publication Number WO 98/07832; and in Achen, *et al.*, *Proc. Nat'l Acad. Sci. U.S.A.*, 95(2): 548-553 (1998), both incorporated herein by reference in the entirety. As explained therein in detail, human VEGF-D is initially produced in human cells as a prepro-VEGF-D polypeptide of 354 amino acids. A cDNA and deduced amino acid sequence for human prepro-VEGF-D are set forth in SEQ ID Nos: 5 and 6, respectively. VEGF-D sequences from other species also have been reported. See Genbank Accession Nos. D89628 (*Mus musculus*); and AF014827 (*Rattus norvegicus*), for example, incorporated herein by reference.

The prepro-VEGF-D polypeptide has a putative signal peptide of 21 amino acids and is apparently proteolytically processed in a manner analogous to the processing of prepro-VEGF-C. A "recombinantly matured" VEGF-D lacking residues 1-92 and 202-354 of SEQ ID NO: 6 retains the ability to activate receptors VEGFR-2 and VEGFR-3, and appears to associate as non-covalently linked dimers. Thus, preferred VEGF-D polynucleotides include those polynucleotides that comprise a nucleotide sequence encoding amino acids 93-201 of SEQ ID NO: 6.

The subject matter of the invention is further described and demonstrated with reference to the following examples.

EXAMPLE 1

Demonstration that hereditary lymphedema is linked to the VEGFR-3 locus

The following experiments, conducted to identify a gene or genes contributing to susceptibility to develop lymphedema, demonstrated that hereditary

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lymphedema correlates, in at least some families, to the chromosomal locus for the VEGFR-3 gene.

OVERVIEW

Families with inherited lymphedema were identified for the purpose of conducting a linkage and positional candidate gene analysis. Thirteen distinct families from the United States and Canada were identified through referrals from lymphedema treatment centers, lymphedema support groups, and from internet correspondence (worldwide web site at www.pitt.edu/~genetics/lymph/) The study protocol was approved by the Institutional Review Board of the University of Pittsburgh and participants gave written informed consent. All members of the families were of western European ancestry. Forty members of one family ("Family 101") were examined during a family reunion by a physiatrist experienced in lymphedema treatment. Family members were considered affected with hereditary lymphedema if they exhibited asymmetry or obvious swelling of one or both legs. Members of the other 12 families were scored as affected if they had received a medical diagnosis of lymphedema, or if there were personal and family reports of extremity swelling or asymmetry. Medical records were obtained to verify status whenever possible. For the purpose of linkage analysis, individuals with very mild or intermittent swelling, heavyset legs, obesity, or a history of leg infections as the only symptom were considered to have indeterminate disease status.

In the 13 families, 105 individuals were classified as affected, with a male:female ratio of 1:2.3. The age of onset of lymphedema symptoms ranged from prenatal (diagnosed by ultrasound) to age 55. When affected by normal matings were analyzed, 76 of 191 children were affected, yielding a penetrance of 80%. First degree relatives of affected individuals were considered at risk.

Biological samples were obtained from members of the thirteen families to conduct the genetic analyses. DNA was isolated from the EDTA-anticoagulated whole blood by the method of Miller *et al.*, *Nucleic Acids Res.*, 16: 1215 (1998), and from cytobrush specimens using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). Analysis of the markers used in the genome scan were performed by methods recognized in the art. [See Browman *et al.*, *Am. J. Hum. Genetic.*, 63:861-869 (1998); see also the NHLBI Mammalian Genotyping Service world-wide web sites

(www.marshmed.org/genetics/methods/pcr.htm; and
www.marshmed.org/genetics/methods/gel.htm).

Two-point linkage analysis was conducted using an autosomal dominant model predicting 80% penetrance in the heterozygous state, 99% penetrance in the homozygous state, and a 1% phenocopy rate. The frequency of the disease allele was set at 1/10,000. Microsatellite marker allele frequencies were calculated by counting founder alleles, with the addition of counts of non-transmitted alleles. Multipoint analysis was carried out using distances obtained from the Location Database (LDB-<http://cedar.genetics.soton.ac.uk/public.html>). Multipoint and 2-point analyses were facilitated using the VITESSE (v1.1) program. [O'Connell, J.R. and Weeks, D.E., (1995), *Nature Genet.*, 11:402-408].

DETAILED DESCRIPTION OF METHODS AND RESULTS

The first family studied, Family 101, was a large, multi-generational family demonstrating early onset lymphedema. (See Fig. 1.) Forty individuals of this family were examined and DNA sampled. In addition, blood was obtained from another 11 members from mailing kits. Linkage simulation was performed using SLINK [Weeks *et al.*, *Am. J. Hum. Genet.* 47:A204 (1990)] and linkage was analyzed using MSIM [Ott, J., *Proc. Nat. Acad. Sci. USA*, 86:4175-4178 (1989)] to estimate the potential power of two point linkage analysis in the family. Marker genotypes were simulated for a marker with heterozygosity of 0.875 under a linked ($\theta=0$) and unlinked ($\theta=0.5$) model using the 51 available individuals. The simulation showed that the power to detect linkage was greater than 90% for a LOD score threshold of $Z(\theta)$ 2.0. The false positive rate was less than 5%.

Shortly thereafter, two additional families (designated Families 106 and 111) segregating for autosomal dominant lymphedema were identified. These three families (Figures 1A-1C, Families 101, 106 and 111) were genotyped for 366 autosomal markers by the NHLBI Mammalian Genotyping Service (www.marshmed.org/genetics). Genotypes were checked for consistency using Pedcheck [O'Connell, J.R. and Weeks, D.E., *Am. J. Hum. Genet.*, 61:A288 (1997)]. Two point linkage analysis was performed using VITESSE [O'Connell, J.R. and Weeks, D.E., *Nature Genet.*, 11:402-408 (1995)]. The model for linkage assumed an

autosomal dominant model of inheritance, a disease allele frequency of 0.0001 and a penetrance of 0.80.

The results from the genomic scan can be briefly summarized as follows. A summed LOD score of greater than 4.0 was observed from distal chromosome 5, markers *D5S1456*, *D5S817* and *D5S488*. The markers on distal chromosome 5q were the only markers having $Z > 3.0$, the criteria established for statistical significance. LOD scores greater than 2.0 ($\theta = 0-0.15$) were also detected for chromosome 12 (*D12S391* $Z = 2.03$, all families), and chromosome 21 (*D21S1440* $Z = 2.62$, all families). The largest two-point LOD ($Z = 4.3$; $\theta = 0$) was observed for marker *D5S408*, localized to chromosome 5q34-q35.

This initial chromosomal mapping was further refined by genotyping the three affected families for eight additional markers localized to region 5q34-q35. Six of these were informative for linkage (*D5S653*, *D5S498*, *D5S408*, *D5S2006*, *D5S1353* and *D5S1354*). Linkage analysis of these markers using VITESSE yielded a 2-point LOD score of 6.1 at $\theta = 0$ for marker *D5S1354* (Table 1) and a maximum multipoint LOD score of 8.8 at marker *D5S1354* (Fig. 2). These findings supported the localization of a gene within chromosome band 5q34-q35 that is a predisposing factor in hereditary lymphedema.

TABLE 1

LOD scores for individual families estimated over the interval defined by markers *D5S498* and *D5S2006*.

	$Z(\theta) 0.0$	$Z(\theta) 0.01$	$Z(\theta) 0.05$	$Z(\theta) 0.1$	$Z(\theta) 0.2$
<i>Locus D5S498</i>					
Family 101	-3.18	-2.33	-0.45	0.42	0.88
Family 106	1.08	1.07	1.05	0.99	0.81
Family 111	-0.85	-0.77	-0.53	-0.34	-0.13
Family 105	1.22	1.20	1.11	0.98	0.72
Family 135	-2.48	-1.85	-1.12	-0.75	-0.38

	$Z(\theta) 0.0$	$Z(\theta) 0.01$	$Z(\theta) 0.05$	$Z(\theta) 0.1$	$Z(\theta) 0.2$
<i>Locus D5S1353</i>					
Family 101	-2.99	-2.48	-1.21	-0.63	-0.18
Family 106	0.28	0.29	0.35	0.38	0.38
Family 111	-1.06	-1.02	-0.88	-0.72	-0.42
Family 105	0.72	0.71	0.65	0.56	0.39
Family 135	-8.03	-4.18	-2.09	-1.13	-0.30
<i>Locus D5S1354</i>					
Family 101	6.09	6.02	5.69	5.21	4.07
Family 106	1.42	1.40	1.32	1.20	0.96
Family 111	0.21	0.22	0.23	0.24	0.22
Family 105	0.43	0.42	0.40	0.36	0.28
Family 135	-6.88	-4.91	-3.20	-2.16	-1.07
<i>Locus D5S408</i>					
Family 101	2.80	2.74	2.50	2.20	1.56
Family 106	0.66	0.68	0.73	0.76	0.71
Family 111	-1.70	-1.40	-0.80	-0.44	-0.10
Family 105	0.42	0.41	0.38	0.35	0.27
Family 135	-5.22	-4.24	-2.58	-1.67	-0.80
<i>Locus D5S2006</i>					
Family 101	4.51	4.70	4.85	4.66	3.80
Family 106	1.17	1.16	1.11	1.03	0.83
Family 111	-1.32	-1.18	-0.82	-0.56	-0.25
Family 105	0.43	0.42	0.40	0.36	0.28
Family 135	-3.86	-3.20	-2.11	-1.45	-0.73

25

During the completion of the genome scan, an additional ten lymphedema families were ascertained. Two of these families (Families 105 and 135, see Figures 1E and 1D), were potentially informative for linkage and were genotyped for markers in the linked region. Examination of the two point LOD scores for the five informative families for markers in the linked region (Table 1) shows that four of the

families (101, 105, 106 and 111) are consistent with linkage to chromosome 5q while family 135 excluded linkage across the entire region with LOD scores $Z \leq -2.0$ for all markers. Multipoint linkage analysis of Families 101, 105, 106 and 111 (Fig. 2) yielded a peak LOD score of $Z = 10$ at marker *D5S1354*. These findings support the existence of at least two loci which predispose to hereditary lymphedema.

The order of markers *D5S1353*, *D5S1354* and *D5S408* with respect to each other was uncertain. Multipoint linkage analysis using alternative orders for these markers gave similar results. Marker *D5S498* is a framework marker and marker *D5S408* is mapped 11.2 centimorgans distal to *D5S498*, based on the CHLC chromosome 5 sex averaged, recombination minimized map, version 3 (www.chlc.org). The physical distance between *D5S498* and *D5S408* is estimated as 1.45 megabases based on the Genetic Location Database (LDB) chromosome 5 summary map (cedar.genetics.soton.ac.uk/public_html/).

Database analysis identified sixteen genes within this region. Two of these genes have been identified as having roles in development (*MSX2* and *VEGFR-3*). *MSX2* was considered an unlikely candidate gene for lymphedema because of its known involvement in craniofacial development [Jabs *et al.*, *Cell*, 75: 443-450 (1993)]. *VEGFR-3*, the gene encoding a receptor for VEGF-C, was selected as a better candidate gene for initial further study for the following reasons.

- (1) *VEGFR-3* is expressed in developing lymphatic endothelium in the mouse [Kukk *et al.*, *Development*, 122: 3829-3837 (1996); and Kaipainen *et al.*, *Proc. Nat. Acad. Sci. USA*, 92: 3566-3570 (1995)];
- (2) expression of *VEGFR-3* is induced in differentiating avian chorioallantoic membrane [Oh *et al.*, *Dev. Biol.*, 188:96-109 (1997)]; and
- (3) overexpression of VEGF-C, a ligand of *VEGFR-3*, leads to hyperplasia of the lymphatic vessels in transgenic mice [Jeltsch *et al.*, *Science*, 276: 1423-1425 (1997)].

To explore the potential role of *VEGFR-3* in lymphedema, probands from the thirteen lymphedema families were screened for variation by direct sequencing of portions of the *VEGFR-3* gene. The sequencing strategy used amplification primers generated based upon the *VEGFR-3* cDNA sequence (SEQ ID NO: 1) and information on the genomic organization of the related vascular endothelial

growth factor receptor-2 (*VEGFR-2/KDR/flk-1*) [Yin *et al.*, *Mammalian Genome*, 9: 408-410 (1998)]. Variable positions (single nucleotide polymorphisms), the unique sequence primers used to amplify sequences flanking each variable site, and the method of detecting each variant are summarized in Table 2.

TABLE 2

Location, amplification primer sequences, amplification conditions, and detection methods for five intragenic single nucleotide polymorphisms in the human VEGFR-3 gene

Position in VEGFR-3 gene	Primer 1 sequence	Primer 2 sequence	Ann. temp.	[MgCl ₂]	Base change	Detection Method
Exon 12, amino acid 641	tcaccatcgatccaagc (SEQ ID NO: 7)	agttctgcgtgagccgag (SEQ ID NO: 8)	56 °C	1.0 mM	C-T	Sequencing
Exon 24, amino acid 1114	caggacggggtgactga (SEQ ID NO: 9)	gccaggcctgtctactg (SEQ ID NO: 10)	56 °C	1.0 mM	C-T	Sequencing
Exon 3, amino acid 175	ccagctcctacgtgttcg (SEQ ID NO: 11)	ggcaacagctggatgtca (SEQ ID NO: 12)	56 °C	1.0 mM	C-T	<i>HhaI</i>
65bp 3' to Exon 6	ctgtgagggcgtgggagt (SEQ ID NO: 13)	gtcctttgagccactgga (SEQ ID NO: 14)	54 °C	1.5 mM	G-A	<i>SstI</i>
55bp 3' to Exon 2	cacacgtcatcgacaccggtg (SEQ ID NO: 15)	ggcaacagctggatgtca (SEQ ID NO: 16)	56 °C	1.5 mM	C-T	<i>ApaI</i>

All amplifications were done for 35 cycles with denaturation at 94° for 30 seconds, annealing as above for 30 seconds, and extension at 72° for 30 seconds.

Amplification and sequencing primers were synthesized by the DNA Synthesis Facility, University of Pittsburgh. Amplification primers were tagged at the 5' end with the forward or reverse M13 universal sequence to facilitate direct sequencing. Amplimers were subjected to cycle sequencing using the dRhodamine terminator ready reaction kit or the Dye Primer ready reaction kit for -M13 and M13 Rev primers (Perkin Elmer) and analyzed on the Prism ABI 377 fluorescent sequencer. Sequences were aligned for further analysis using SEQUENCHER 3.0 (Gene Codes).

Genomic sequence from approximately 50% of the VEGFR-3 gene was determined in this manner, and five single nucleotide variants were observed. Two of the variants occurred in introns, and a third was a silent substitution in predicted exon 3. These intragenic polymorphisms were used to map the *VEGFR-3* gene. As shown in Figure 2, *VEGFR-3* maps within the region of chromosome 5q linked to the lymphedema phenotype, consistent with it being selected as a candidate gene. In two

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families, (Family 127, pedigree not shown, and Family 135), a C-T transition was identified at nucleotide position 1940 of the *VEGFR-3* cDNA (SEQ ID NO: 1). This nucleotide substitution is predicted to lead to a non-conservative substitution of serine (codon TCC) for proline (codon CCC) at residue 641 (putative exon 12, within the sixth immunoglobulin-like region of the receptor's extracellular domain) of the amino acid sequence of the receptor (SEQ ID NO: 2). However, this sequence change was observed in 2 of 120 randomly selected individuals from the general population (240 alleles). Also, in one of the two families in which this variant was initially detected, family 135, linkage between lymphedema and chromosome 5q markers was excluded (Table 1 and Figure 2). In probands from the other ten families, wild type sequence was observed at nucleotide position 1940. Collectively, these results suggest that this P641S variant is not causative.

In one nuclear family (Family 127, pedigree shown in Figure 1F) a C-T transition was observed at nucleotide position 3360 (SEQ ID NO: 1) of the *VEGFR-3* cDNA. This nucleotide substitution is predicted to lead to a non-conservative substitution of leucine (codon CTG) for proline (codon CCG) at residue 1114 of the amino acid sequence of the receptor (SEQ ID NO: 2). This P1114L mutation is predicted to lie in the intracellular tyrosine kinase domain II involved in intracellular signaling [Pajusola *et al.*, *Cancer Res.*, 52:5738- 5743 (1992)]. Direct sequencing of predicted exon 20 of the *VEGFR-3* gene alleles from members of this family identified this substitution only in affected and at-risk family members. This sequence change was not observed in 120 randomly selected individuals of mixed European ancestry from the general population (240 alleles). In probands from the other 11 families, wild type sequence was observed at nucleotide position 3360.

Collectively, this data demonstrates that a missense mutation that causes a non-conservative substitution in a kinase domain of the *VEGFR-3* protein correlates strongly with a heretable lymphedema in one family, and suggests that other mutations in the same gene may exist that correlate with heretable lymphedema in other families. As explained above, only a portion of the *VEGFR-3* gene sequence was analyzed to identify this first mutation of interest. Additional sequencing, using standard techniques and using the known *VEGFR-3* gene sequence for guidance, is expected to identify additional mutations of interest that are observed in affected and at-risk members of other families studied.

EXAMPLE 2**Demonstration that a C-T missense mutation at position 3360 in the VEGFR-3 coding sequence results in a tyrosine kinase negative mutant**

The results set forth in Example 1 identified two missense mutations in the *VEGFR-3* coding sequence, one of which (C-T at position 3360) appeared to correlate with heritable lymphedema and one of which (C-T transition at position 1940) did not. The following experiments were conducted to determine the biochemical significance of these mutations on VEGFR-3 biological activity.

To analyze how the two single amino acid substitutions affect the VEGFR-3-mediated signaling, the corresponding mutant receptor expression vectors were generated using site-directed mutagenesis procedures and expressed in 293T cells by transient transfection. The long form of human VEGFR-3 cDNA (SEQ ID NO: 1) was cloned as a *Hind* III-*Bam* HI fragment from the LTR-FLT4I plasmid [Pajusola *et al.*, *Oncogene* 8: 2931-2937 (1993)] into pcDNA3.1/Z(+) (Invitrogen). The P641S and P1114L mutants of VEGFR-3 were generated from this construct with the GeneEditor™ *in vitro* Site-Directed Mutagenesis System (Promega) using the following oligonucleotides (the C-T mutations are indicated with bold letters):

5'-CCTGAGTATCT**CT**CCCGCGTCGC-3' (SEQ ID NO: 17) for P641S mutation; and

5'-GGTGCCTCCCT**CT**ACCCTGGG-3' (SEQ ID NO: 18) for P1114L mutation.

For the transient expression studies, 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (GIBCO BRL, Life Technologies, Gaithersburg, MD), glutamine, and antibiotics. Cells were transfected with 20 µg of plasmid encoding the wild type or mutant VEGFR-3 forms using the calcium phosphate method, and harvested 36 hours after transfection for immunoprecipitation and Western blotting. Under these conditions, RTK overexpression results in ligand-independent activation, thus allowing the receptor phosphorylation to be studied. An empty vector was used for mock (control) transfections.

In order to investigate the effect of the two VEGFR-3 mutants on the tyrosine phosphorylation of the VEGFR-3, Western blotting analysis was performed using anti-phosphotyrosine antibodies. The cell monolayers were washed three times

with cold phosphate-buffered saline (PBS, containing 2 mM vanadate and 2 mM PMSF) and scraped into RIPA buffer (150 mM NaCl, 1 % Nonidet P40, 0.5 % deoxycholic acid sodium salt, 0.1 % SDS, 50 mM Tris-HCl, pH 8.0) containing 2 mM Vanadate, 2 mM PMSF, and 0.07 U/ml Aprotinin.

5 The cell lysates were sonicated and centrifuged for 10 minutes at 19,000 X g, and the supernatants were incubated for 2 hours on ice with 2 µg/ml of monoclonal anti-VEGFR-3 antibodies (9D9f9) [Jussila *et al.*, *Cancer Res.*, 58: 1599-604 (1998)]. Thereafter, Protein A sepharose (Pharmacia) beads were added and incubation was continued for 45 minutes with rotation at +4°C. The sepharose
10 beads were then washed three times with ice-cold RIPA buffer and twice with PBS (both containing 2 mM vanadate, 2 mM PMSF), analyzed by 7.5 % SDS-PAGE and transferred to a nitrocellulose filter (Protran Nitrocellulose, Schleicher & Schuell, No. 401196) using semi-dry transfer apparatus. After blocking the filter with 5 % BSA in TBS-T buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05 % Tween 20), the filters were
15 incubated with the phosphotyrosine-specific primary antibodies (Upstate Biotechnology, #05-321), followed by biotinylated goat-anti-mouse immunoglobulins (Dako, E0433) and Biotin-Streptavidin HRP complex (Amersham, RPN1051). The bands were visualized by the enhanced chemiluminescence (ECL) method.

 After analysis for phosphotyrosine-containing proteins, the filters were
20 stripped by washing for 30 minutes at +50°C in 100 mM 2-mercaptoethanol, 2 % SDS, 62.5 mM Tris-HCl, pH 6.7, with occasional agitation. The filters were washed with TBS-T, blocked again with BSA as described above, and analyzed for the presence of VEGFR-3 using the 9D9f9 antibodies and HRP-conjugated rabbit-anti-mouse immunoglobulins (Dako, P0161).

25 The Western analyses revealed that the P641S mutant receptor was phosphorylated normally, *i.e.*, in a manner similar to the wild type control. However, the proteolytic processing of the P641S receptor protein may be affected, as the 175 kD and 125 kD polypeptides seemed to have a higher relative density when compared to the 195 kD form.

30 In contrast, no phosphorylated P1114L mutant protein was detected using the phosphotyrosine antibodies. The expression of similar amounts of the VEGFR-3 protein (normal and both mutants) was confirmed using the monoclonal 9D9f9 antibody, which is directed towards the extracellular domain of the VEGFR-3.

Both the P641S and the P1114L mutant VEGFR-3 migrated slightly faster than the wild type VEGFR-3 in the gel electrophoresis.

In order to analyze the possible dominant negative effect of the P1114L mutant on the wild-type receptor, a second, similar set of experiments were performed wherein the 293T cells were transfected with an increasing amount of the P1114L expression vector in combination with decreasing amounts of the wild type vector. Wild type to mutant ratios of 1:0, 3:1, 1:1, 1:3 and 0:1 were used. The cells were lysed 48 hours after transfection and the lysates were analyzed by immunoprecipitation and Western blotting as described above. These experiments permitted evaluation of whether the mutant protein interferes with wild type protein phosphorylation and estimation of the minimal amount of the WT protein needed for observable tyrosyl autophosphorylation. Immunoprecipitates from cells transfected with only the WT plasmid revealed WT protein that was strongly phosphorylated in this experiment (lane 2), whereas immunoprecipitates from cells transfected with only the mutant plasmid were again inactive (unphosphorylated).

Interestingly, when transfection was made using 75% of WT and 25 % of mutant plasmid, the phosphorylation of the receptors was decreased by about 90%. This result strongly suggests that the P1114L mutant receptor forms heterodimers with the WT receptor, but cannot phosphorylate the WT receptor, thus failing to activate it. Under this theory, the WT receptor monomers in the heterodimers would also remain inactive, causing a disproportionate decrease of the total amount of activated receptor, when co-transfected with the mutant. Wildtype-wildtype homodimers would remain active and be responsible for the observed signaling. When the wild type and mutant receptor expression vectors were transfected at a 1:1 ratio, the VEGFR-3 phosphorylation was about 4% of the wild type alone, whereas at a 1:3 ratio, no tyrosine phosphorylation of VEGFR-3 was observed.

The foregoing results are consistent with the linkage analyses in Example 1: the mutation at position 641 that did not appear to correlate with lymphedema also did not appear to be dysfunctional, whereas the mutation at position 1114 appeared to cause a dominant negative mutation that shows no tyrosine phosphorylation alone and that drastically reduces VEGFR-3 signalling in cells expressing both the mutant and wild type VEGFR-3 genes.

Collectively, these data indicate that the P1114L VEGFR-3 mutant is unable to act as a part of the signaling cascade, and also acts in a dominant negative manner, thus possibly interfering partially with the activation of the wild type VEGFR-3. Such effects of the mutation may eventually lead to lymphedema.

5

EXAMPLE 3

Treatment of lymphedema with a VEGFR-3 ligand

The data from Examples 1 and 2 collectively indicate a causative role in heritable lymphedema for a mutation in the *VEGFR-3* gene that interferes with VEGFR-3 signaling. Such a mutation behaves in an autosomal dominant pattern, due to the apparent necessity for receptor dimerization in the signaling process. However, the data from Example 2 suggests that some residual signaling may still occur in heterozygous affected individuals, presumably through pairing of VEGFR-3 proteins expressed from the wild type allele. The following experiments are designed to demonstrate the efficacy of VEGFR-3 ligand treatment in such affected individuals, to raise VEGFR-3 signaling to levels approaching normal and thereby ameliorate/palliate the symptoms of hereditary lymphedema.

Initially, an appropriate animal model is selected. Several potential animal models have been described in the literature. [See, e.g., Lyon *et al.*, *Mouse News Lett.* 71: 26 (1984), *Mouse News Lett.* 74: 96 (1986), and *Genetic variants and strains of the laboratory mouse*, 2nd ed., New York: Oxford University Press (1989), p. 70 (*Chylous ascites* mouse); Dumont *et al.*, *Science*, 282: 946-949 (1998) (heterozygous VEGFR-3 knockout mouse); Patterson *et al.*, "Hereditary Lymphedema," *Comparative Pathology Bulletin*, 3: 2 (1971) (canine hereditary lymphedema model); van der Putte, "Congenital Hereditary Lymphedema in the Pig," *Lympho*, 11: 1-9 (1978); and Campbell-Beggs *et al.*, "Chyloabdomen in a neonatal foal," *Veterinary Record*, 137: 96-98 (1995).] Those models which are determined to have analogous mutations to the VEGFR-3 gene are preferred. In a preferred embodiment, "knock in" homologous recombination genetic engineering strategies are used to create an animal model (e.g., a mouse model) having a VEGFR-3 allelic variation analogous to the human variations described herein. [See, e.g., Partanen *et al.*, *Genes & Development*, 12: 2332-2344 (1998) (gene targeting to introduce mutations into another receptor protein (FGFR-1) in mice).] For example, the P1114L

mutation in human VEGFR-3 occurs in a VEGFR-3 region having highly conserved amino acid identity with murine VEGFR-3 (Genbank Accession No. L07296). Thus, a corresponding P1114L can be introduced into the murine VEGFR-3 by "knock-in" homologous recombination. Optionally, such mice can be bred to the heterozygous VEGFR-3 knockout mice or *Chy* mice described above to further modify the phenotypic severity of the lymphedema disease.

The mice as described above are treated with a candidate therapeutic, e.g., a recombinant mature form of VEGF-C, at various dosing schedules, e.g., once daily by intravenous (IV) or intramuscular (IM) injection at a dose of 1-1000 ng/g body weight, preferably 10-100 ng/g, which should result in a peak level saturating VEGFR-3 (K_d about 150 pM) but not VEGFR-2 (K_d around 400 pM). Direct IM injection at multiple sites in the muscles of affected extremities is a preferred route of administration. The dosing is adjusted according to the efficacy of the treatment and the presence of possible side effects due to the lowering of blood pressure, which has been observed in response to VEGF administration IV. The efficacy of treatment is measured via NMRI imaging of the water content and volume of swelling of the abdomen and the extremities of the animals. The amount of fluid in the abdominal cavity is estimated and the animals are weighed during the follow-up.

In studies using VEGFR-3 $-/+$ x *Chy* mice progeny, the animals will also have the β -galactosidase marker in their lymphatic endothelium. After a successful treatment, the treated and non-treated experimental animals and VEGFR-3 $-/+$ controls are killed and their lymphatic vessels are visualized by β -gal and antibody staining. The staining patterns of experimental and control animals are compared for vessel diameter, numbers of endothelial cells, density of blood and lymphatic vessels, and nuclear density/section surface area for the estimation of tissue oedema.

Such experiments are repeated with various candidate therapeutics (e.g., VEGF-C or VEGF-D recombinant polypeptides; VEGF-C and VEGF-D gene therapy vectors; and combinations thereof) at various dosing schedules to determine an optimum treatment regimen.

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those in the art, all of which are intended as aspects of the present invention. Accordingly, only such limitations as appear in the claims should be placed on the invention.

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CLAIMS

What is claimed is:

1. A method of screening a human subject for an increased risk of developing a lymphatic disorder, comprising the steps of:

5 (a) assaying nucleic acid of a human subject to determine a presence or an absence of a mutation altering the sequence or expression of at least one VEGFR-3 allele; and

(b) screening for an increased risk of developing a lymphatic disorder from the presence or absence of said mutation, wherein the presence of a mutation altering the
10 encoded amino acid sequence or expression of at least one VEGFR-3 allele in the nucleic acid correlates with an increased risk of developing a lymphatic disorder.

2. A method according to claim 1 wherein the assaying step comprises determining the presence or absence of a mutation altering a tyrosine kinase domain amino acid sequence of the protein encoded by the VEGFR-3 allele.

15 3. A method according to claim 1 wherein the assaying step comprises determining the presence or absence of a missense mutation in the VEGFR-3 allele at a position corresponding to codon 1114 of the VEGFR-3 amino acid sequence set forth in SEQ ID NO: 2.

4. A method according to claim 1 wherein the assaying step comprises at least one procedure selected from the group consisting of:

(a) determining a nucleotide sequence of at least one codon of at least one VEGFR-3 allele of the human subject;

5 (b) performing a hybridization assay to determine whether nucleic acid from the human subject has a nucleotide sequence identical to or different from one or more reference sequences;

(c) performing a polynucleotide migration assay to determine whether nucleic acid from the human subject has a nucleotide sequence identical to or different
10 from one or more reference sequences; and

(d) performing a restriction endonuclease digestion to determine whether nucleic acid from the human subject has a nucleotide sequence identical to or different from one or more reference sequences.

5. A method according to claim 1 wherein the assaying step comprises:
15 performing a polymerase chain reaction (PCR) to amplify nucleic acid comprising VEGFR-3 coding sequence, and determining nucleotide sequence of the amplified nucleic acid.

6. A method of screening for a VEGFR-3 hereditary lymphedema genotype in a human patient, comprising the steps of:

20 (a) providing a biological sample comprising nucleic acid from said patient, said nucleic acid including sequences corresponding to said patient's VEGFR-3 alleles;

(b) analyzing said nucleic acid for the presence of a mutation or mutations;

25 (c) determining a VEGFR-3 genotype from said analyzing step; and

(d) correlating the presence of a mutation in a VEGFR-3 allele with a hereditary lymphedema genotype.

7. The method according to claim 6 wherein said biological sample is a cell sample.

8. The method according to claim 6 wherein said analyzing comprises sequencing a portion of said nucleic acid, said portion comprising at least one codon of said VEGFR-3 alleles.

9. The method according to claim 8 wherein said nucleic acid is DNA.

5 10. The method according to claim 8 wherein said nucleic acid is RNA.

11. A method of treatment for hereditary lymphedema, comprising the step of administering to a patient with hereditary lymphedema a therapeutically effective amount of a growth factor product selected from the group consisting of vascular endothelial growth factor C (VEGF-C) protein products, vascular endothelial growth factor D (VEGF-D) protein products, VEGF-C gene therapy products, and VEGF-D gene therapy protein products.

12. A therapeutic or prophylactic method of treating lymphedema, comprising the steps of:

15 providing isolated lymphatic endothelial cells or lymphatic endothelial progenitor cells;

transforming or transfecting the cells *ex vivo* with a polynucleotide comprising a nucleotide sequence that encodes a wild type VEGFR-3;

and administering the transformed or transfected cells to the mammalian subject.

20 13. An oligonucleotide useful as a probe for identifying polymorphisms in a human Flt4 receptor tyrosine kinase gene, the oligonucleotide comprising 6-50 nucleotides that have a sequence that is identical or exactly complementary to a portion of a human VEGFR-3 gene sequence or VEGFR-3 coding sequence, except for one sequence difference selected from the group consisting of a nucleotide addition, a nucleotide deletion, or nucleotide substitution.

25

14. An oligonucleotide according to claim 13 wherein the nucleotide sequence is exactly identical or exactly complementary to a portion of the human VEGFR-3 coding sequence set forth in SEQ ID NO: 1, except for a nucleotide substitution at a position corresponding to nucleotide 3360 of SEQ ID NO: 1.

5 15. A kit comprising at least two oligonucleotides of the formula X_nYZ_m or its complement;

where n and m are integers from 0 to 49;

where $5 \leq (n + m) \leq 49$;

10 where X_n is a stretch of n nucleotides identical to a first portion of SEQ ID NO: 1, said first portion ending immediately upstream (5') of position 3360 of SEQ ID NO: 1; and

where Z_m is a stretch of m nucleotides identical to a second portion of SEQ ID NO: 1, said second portion beginning immediately downstream (3') of position 3360 of SEQ ID NO: 1; and

15 wherein Y represents a nucleotide selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil nucleotides.

20 16. An array of oligonucleotide probes immobilized on a solid support, wherein each probe occupies a separate known site in the array; and wherein the array includes at least one probe set comprising two to four probes, wherein one probe is exactly identical or exactly complementary to a human VEGFR-3 coding sequence, and the other one to three members of the set are exactly identical to the first member, but for at least one different nucleotide, which different nucleotide is located in the same position in each of the one to three additional set members.

17. A purified polynucleotide comprising a nucleotide sequence encoding a human VEGFR-3 protein variant, wherein said polynucleotide is capable of hybridizing to the complement of SEQ ID NO: 1 under the following hybridization conditions:
hybridization at 42°C in 50% formamide, 5X SSC, 20 mM Na•PO₄, pH 6.8;
5 and
washing in 0.2X SSC at 55°C;
and wherein the encoded VEGFR-3 protein variant has an amino acid sequence that differs at position 1114 from the amino acid sequence set forth in SEQ ID NO: 1.
18. A purified polynucleotide comprising a nucleotide sequence encoding a
10 VEGFR-3 protein of a human that is affected with heritable lymphedema;
wherein said polynucleotide is capable of hybridizing to the complement of SEQ ID NO: 1 under the following hybridization conditions: hybridization at 42°C in 50% formamide, 5X SSC, 20 mM Na•PO₄, pH 6.8; and washing in 0.2X SSC at 55°C;
15 and wherein the encoded polynucleotide has an amino acid sequence that differs from SEQ ID NO: 1 at at least one codon.
19. A vector comprising a polynucleotide according to claim 18.
20. A host cell that has been transformed or transfected with a
polynucleotide according to claim 18 and that expresses the VEGFR-3 protein
20 encoded by the polynucleotide.
21. A host cell according to claim 20 that has been co-transfected with a polynucleotide encoding the VEGFR-3 amino acid sequence set forth in SEQ ID NO: 2 and that expresses the VEGFR-3 protein having the amino acid sequence set forth in SEQ ID NO: 2.

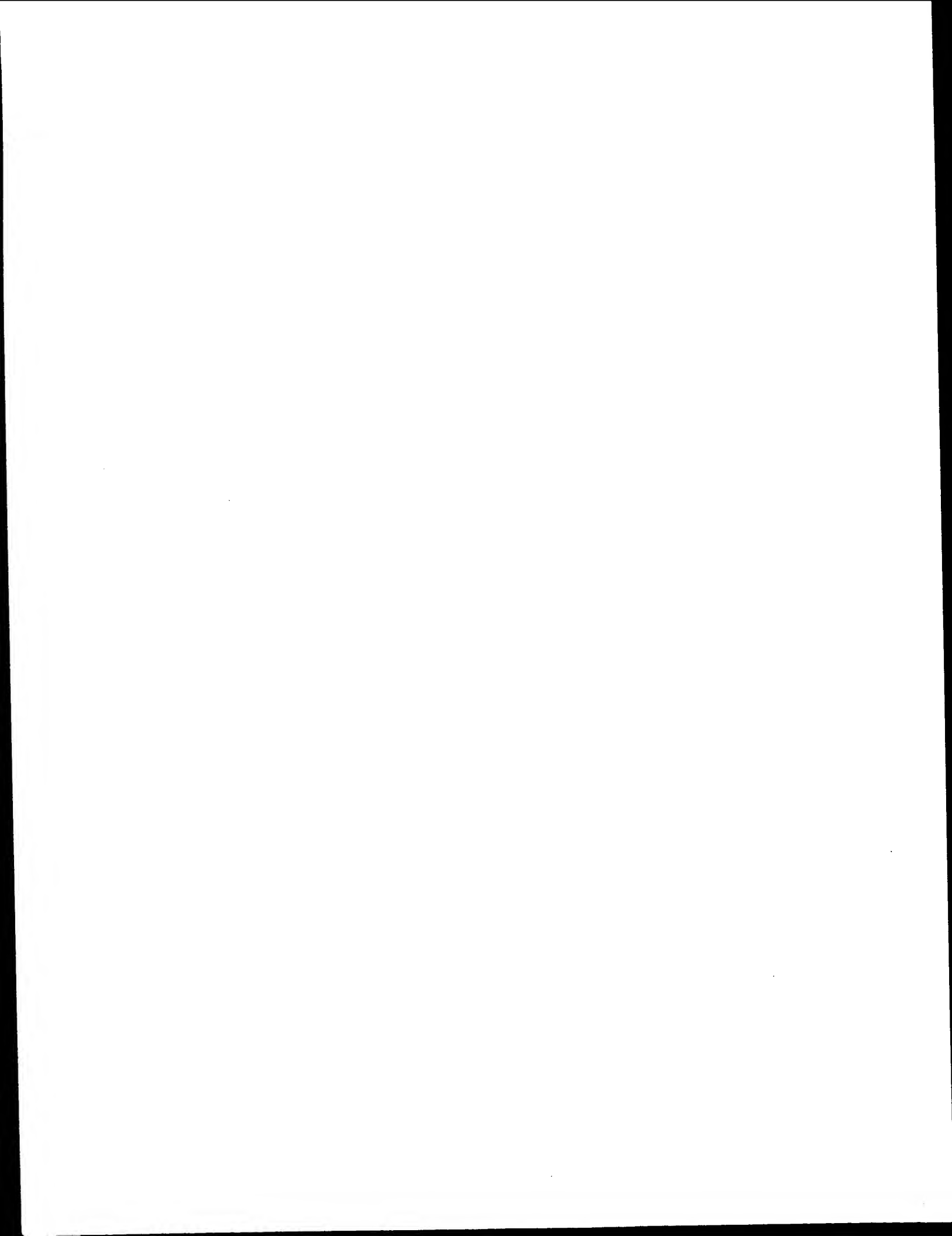
22. A method for identifying a modulator of intracellular VEGFR-3 signaling, comprising the steps of:

- 5 a) contacting a cell expressing at least one mutant mammalian VEGFR-3 polypeptide in the presence and in the absence of a putative modulator compound;
- b) detecting VEGFR-3 signaling in the cell; and
- c) identifying a putative modulator compound in view of decreased or increased signaling in the presence of the putative modulator, as compared to signaling in the absence of the putative modulator.

10 23. A method according to claim 22 wherein the cell expresses the mutant mammalian VEGFR-3 polypeptide and a wildtype mammalian VEGFR-3 polypeptide.

24. A method according to claim 23 wherein the mutant and wildtype VEGFR-3 polypeptides are human.

15 25. A method according to claim 24 wherein the mutant VEGFR-3 polypeptide comprises a leucine amino acid at the position corresponding to position 1114 of SEQ ID NO: 1.



- 1 -

SEQUENCE LISTING

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 Alitalo, Kari
 Finegold, David N
 Karkkainen, Marika

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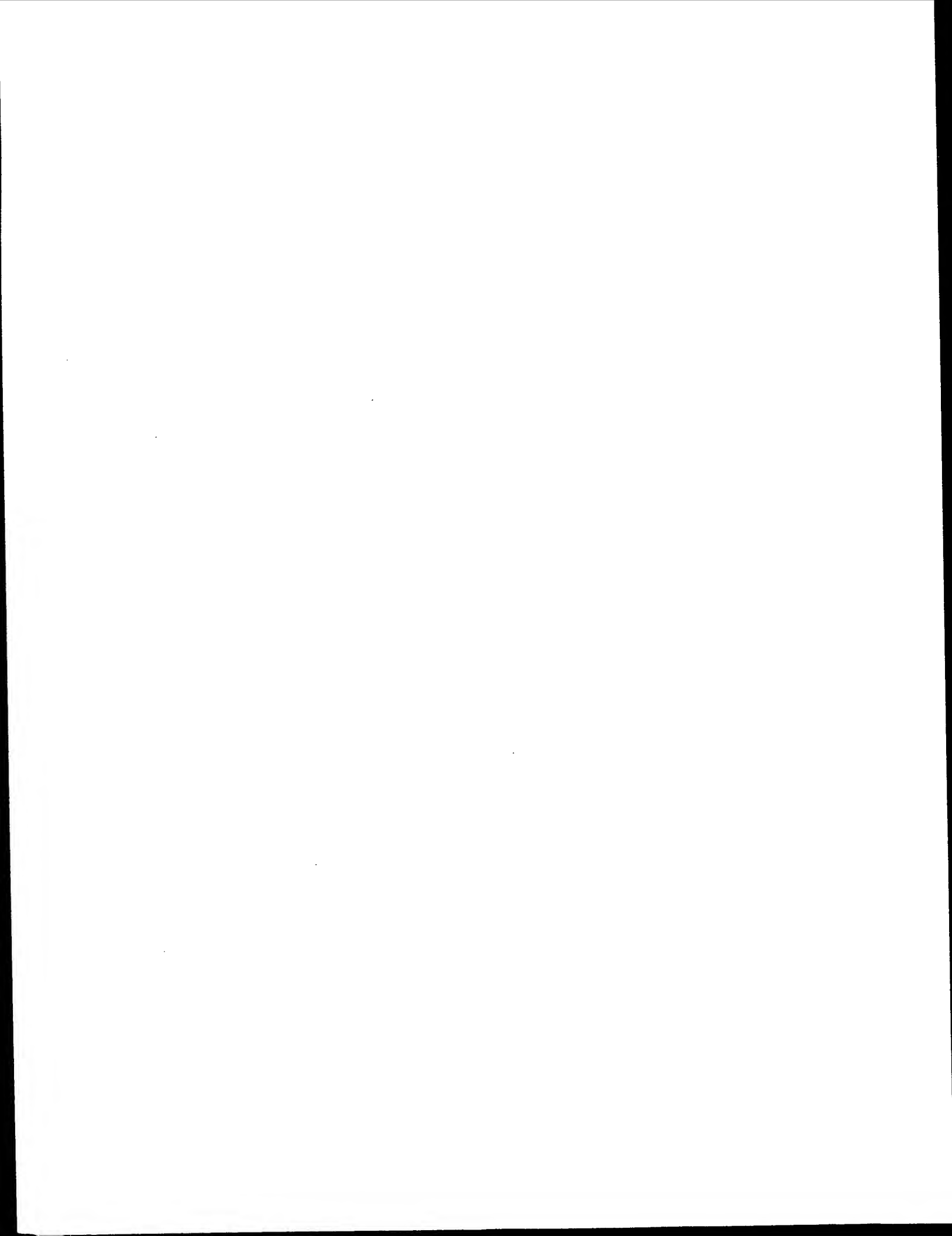
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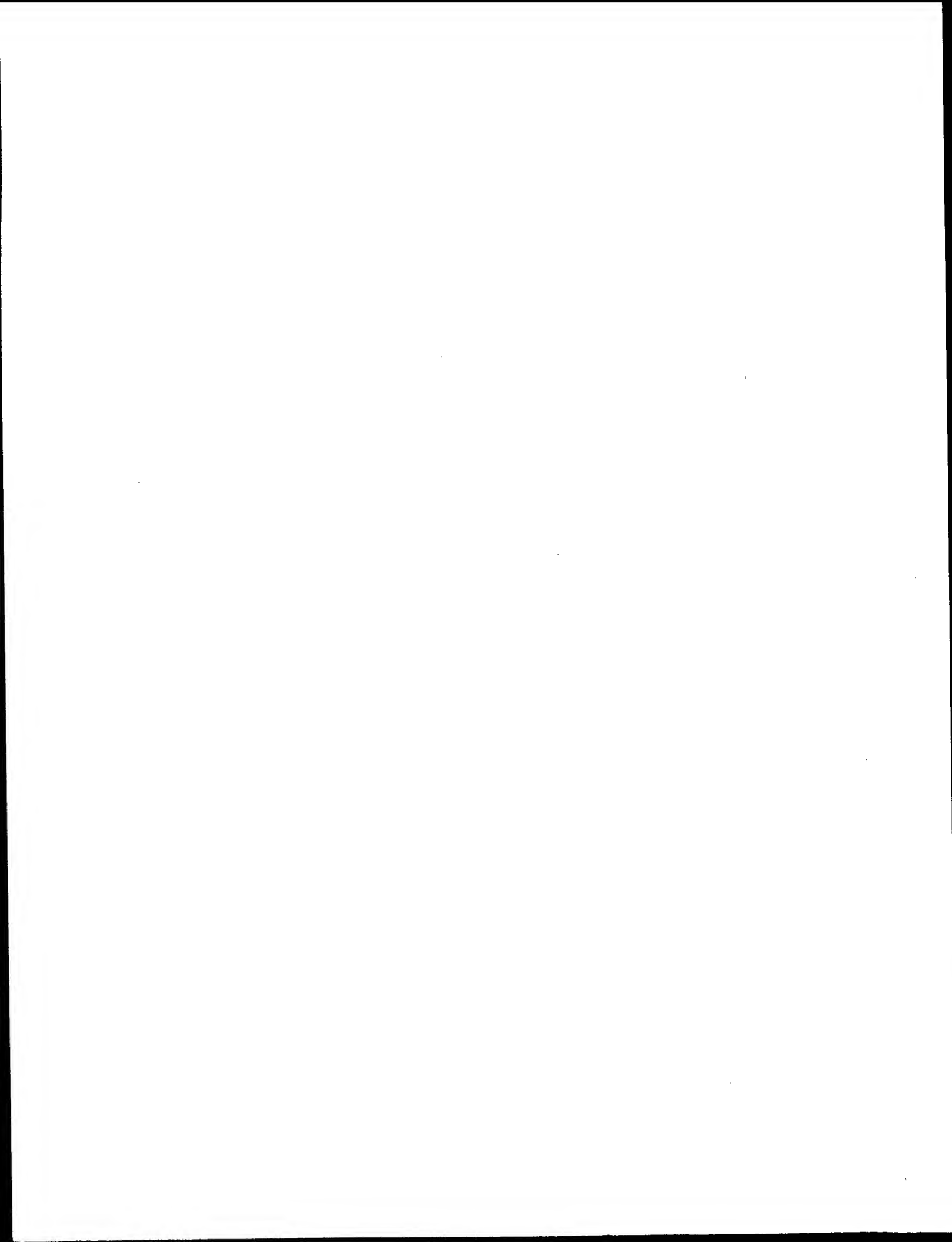

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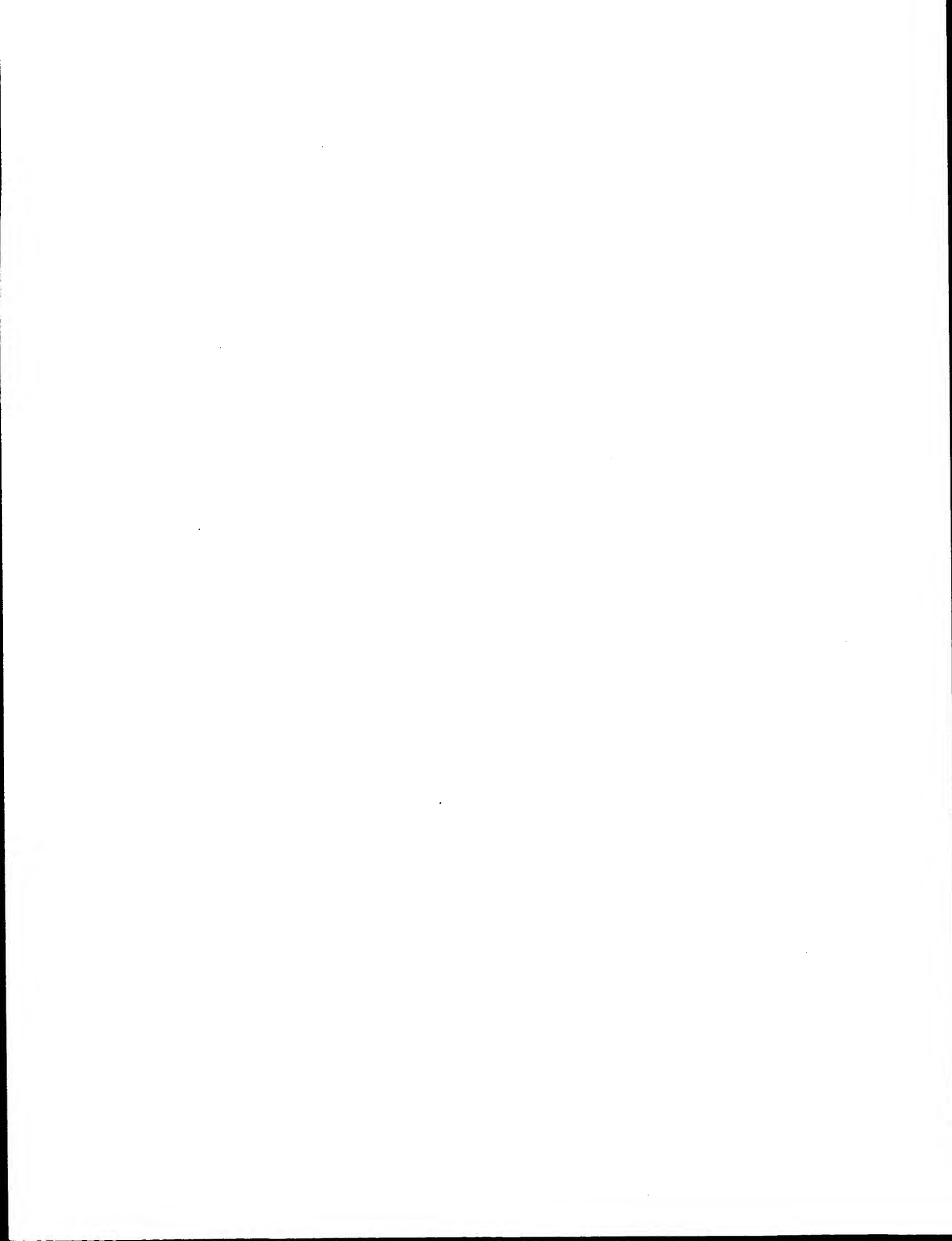
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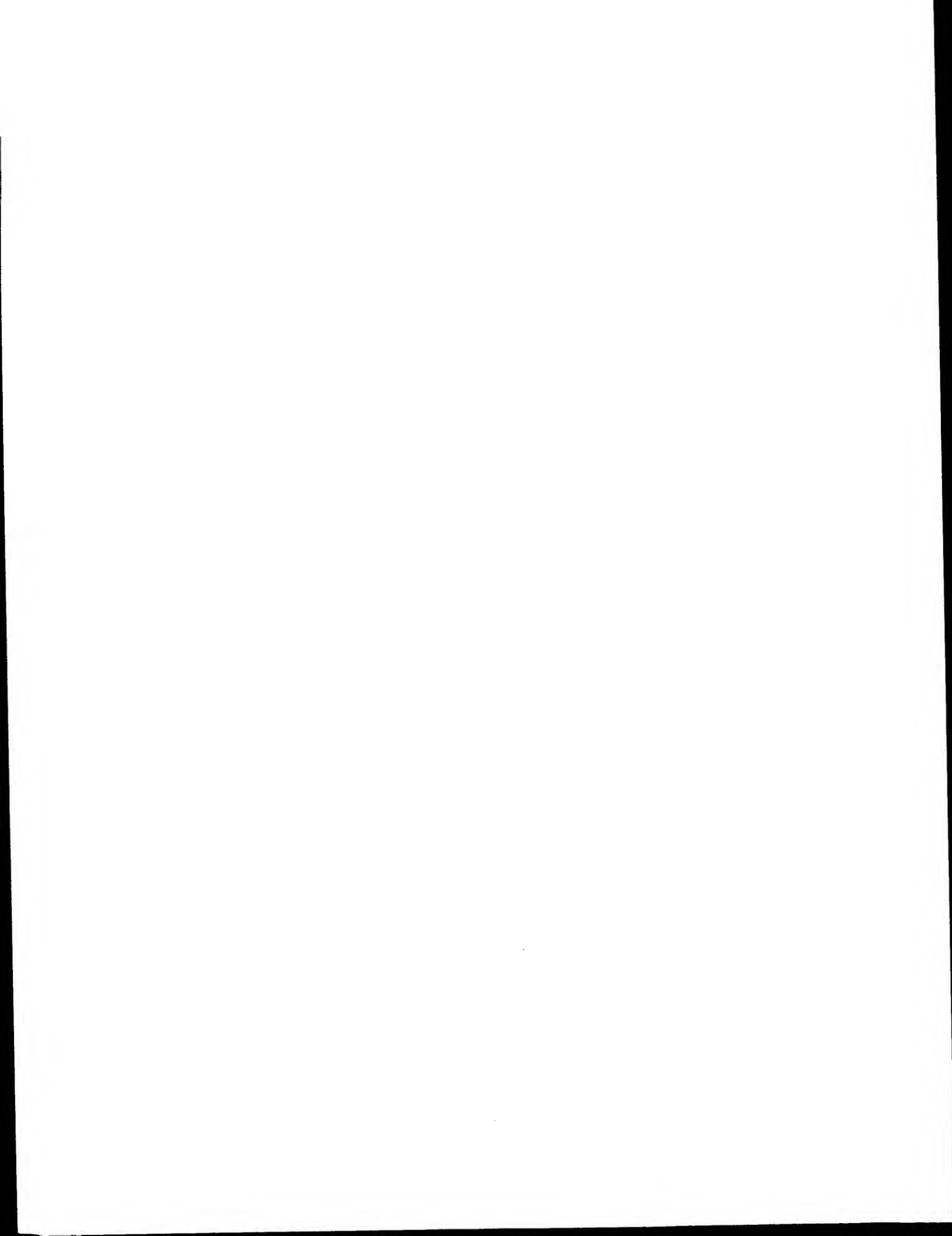
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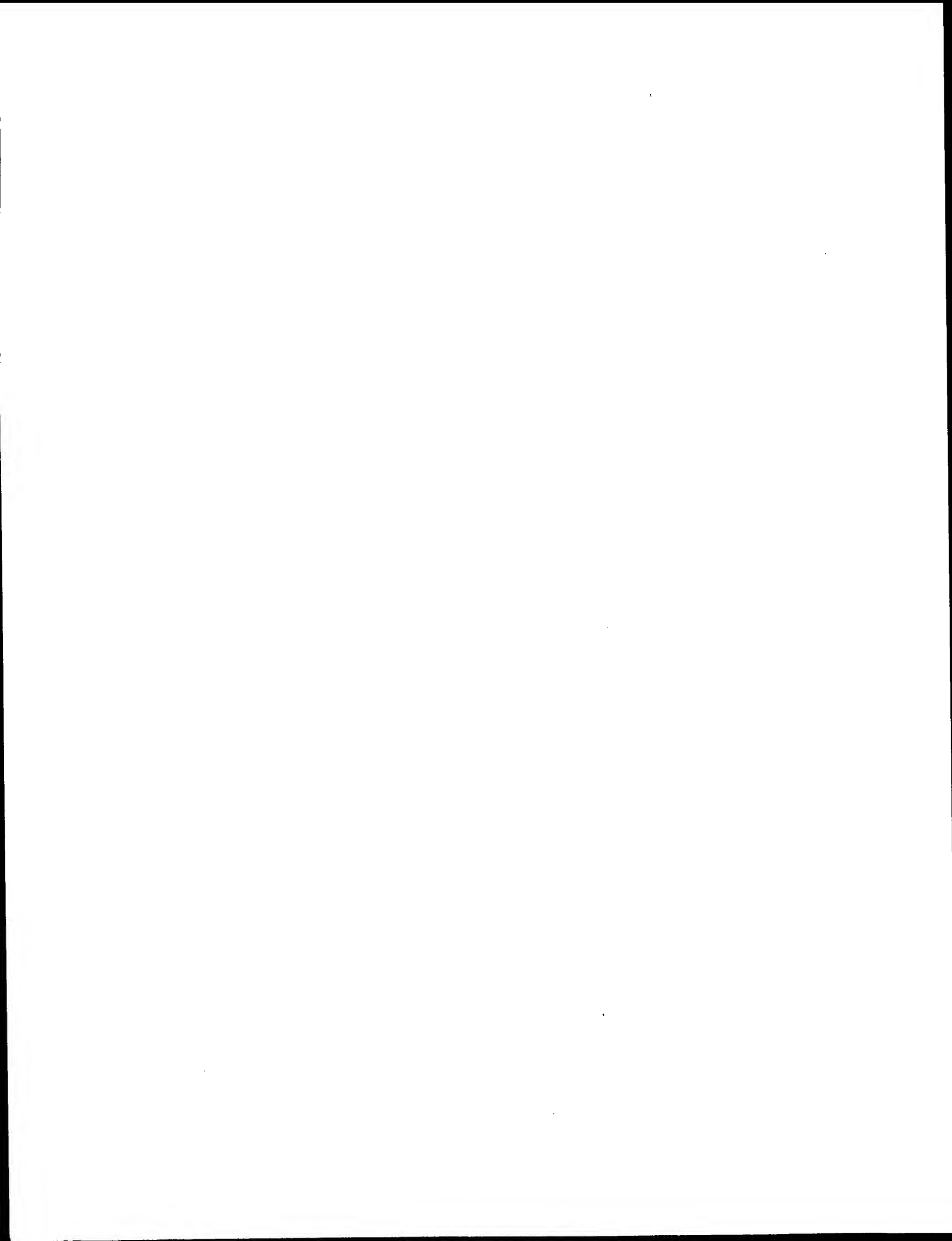
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 Lys Ser Gly Val Asp Leu Ala Asp Ser Asn Gln Lys Leu Ser Ile Gln
 725 730 735
 Arg Val Arg Glu Glu Asp Ala Gly Arg Tyr Leu Cys Ser Val Cys Asn
 740 745 750
 Ala Lys Gly Cys Val Asn Ser Ser Ala Ser Val Ala Val Glu Gly Ser
 755 760 765
 Glu Asp Lys Gly Ser Met Glu Ile Val Ile Leu Val Gly Thr Gly Val
 770 775 780
 Ile Ala Val Phe Phe Trp Val Leu Leu Leu Leu Ile Phe Cys Asn Met
 785 790 795 800
 Arg Arg Pro Ala His Ala Asp Ile Lys Thr Gly Tyr Leu Ser Ile Ile
 805 810 815
 Met Asp Pro Gly Glu Val Pro Leu Glu Glu Gln Cys Glu Tyr Leu Ser
 820 825 830
 Tyr Asp Ala Ser Gln Trp Glu Phe Pro Arg Glu Arg Leu His Leu Gly
 835 840 845
 Arg Val Leu Gly Tyr Gly Ala Phe Gly Lys Val Val Glu Ala Ser Ala
 850 855 860
 Phe Gly Ile His Lys Gly Ser Ser Cys Asp Thr Val Ala Val Lys Met
 865 870 875 880
 Leu Lys Glu Gly Ala Thr Ala Ser Glu His Arg Ala Leu Met Ser Glu
 885 890 895
 Leu Lys Ile Leu Ile His Ile Gly Asn His Leu Asn Val Val Asn Leu
 900 905 910
 Leu Gly Ala Cys Thr Lys Pro Gln Gly Pro Leu Met Val Ile Val Glu
 915 920 925
 Phe Cys Lys Tyr Gly Asn Leu Ser Asn Phe Leu Arg Ala Lys Arg Asp
 930 935 940
 Ala Phe Ser Pro Cys Ala Glu Lys Ser Pro Glu Gln Arg Gly Arg Phe
 945 950 955 960

- 11 -

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 965 970 975
 Ser Asp Arg Val Leu Phe Ala Arg Phe Ser Lys Thr Glu Gly Gly Ala
 980 985 990
 Arg Arg Ala Ser Pro Asp Gln Glu Ala Glu Asp Leu Trp Leu Ser Pro
 995 1000 1005
 Leu Thr Met Glu Asp Leu Val Cys Tyr Ser Phe Gln Val Ala Arg Gly
 1010 1015 1020
 Met Glu Phe Leu Ala Ser Arg Lys Cys Ile His Arg Asp Leu Ala Ala
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 Arg Asn Ile Leu Leu Ser Glu Ser Asp Val Val Lys Ile Cys Asp Phe
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 Gly Leu Ala Arg Asp Ile Tyr Lys Asp Pro Asp Tyr Val Arg Lys Gly
 1060 1065 1070
 Ser Ala Arg Leu Pro Leu Lys Trp Met Ala Pro Glu Ser Ile Phe Asp
 1075 1080 1085
 Lys Val Tyr Thr Thr Gln Ser Asp Val Trp Ser Phe Gly Val Leu Leu
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 Trp Glu Ile Phe Ser Leu Gly Ala Ser Pro Tyr Pro Gly Val Gln Ile
 105 1110 1115 1120
 Asn Glu Glu Phe Cys Gln Arg Leu Arg Asp Gly Thr Arg Met Arg Ala
 1125 1130 1135
 Pro Glu Leu Ala Thr Pro Ala Ile Arg Arg Ile Met Leu Asn Cys Trp
 1140 1145 1150
 Ser Gly Asp Pro Lys Ala Arg Pro Ala Phe Ser Glu Leu Val Glu Ile
 1155 1160 1165
 Leu Gly Asp Leu Leu Gln Gly Arg Gly Leu Gln Glu Glu Glu Val
 1170 1175 1180
 Cys Met Ala Pro Arg Ser Ser Gln Ser Ser Glu Glu Gly Ser Phe Ser
 185 1190 1195 1200
 Gln Val Ser Thr Met Ala Leu His Ile Ala Gln Ala Asp Ala Glu Asp
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 Ser Pro Pro Ser Leu Gln Arg His Ser Leu Ala Ala Arg Tyr Tyr Asn
 1220 1225 1230
 Trp Val Ser Phe Pro Gly Cys Leu Ala Arg Gly Ala Glu Thr Arg Gly
 1235 1240 1245
 Ser Ser Arg Met Lys Thr Phe Glu Glu Phe Pro Met Thr Pro Thr Thr
 1250 1255 1260

- 12 -

Tyr Lys Gly Ser Val Asp Asn Gln Thr Asp Ser Gly Met Val Leu Ala
 265 1270 1275 1280

Ser Glu Glu Phe Glu Gln Ile Glu Ser Arg His Arg Gln Glu Ser Gly
 1285 1290 1295

Phe Ser Cys Lys Gly Pro Gly Gln Asn Val Ala Val Thr Arg Ala His
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Pro Asp Ser Gln Gly Arg Arg Arg Arg Pro Glu Arg Gly Ala Arg Gly
 1315 1320 1325

Gly Gln Val Phe Tyr Asn Ser Glu Tyr Gly Glu Leu Ser Glu Pro Ser
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 ggaacgcgga gccccggacc cgctcccgcc gcctccggct cgcccagggg gggtcgcccg 240
 gaggagcccc ggggagaggg accaggaggg gccgcgggcc tcgcaggggc gccgcgccc 300
 ccacccctgc ccccgccagc ggaccgggtcc cccacccccg gtccttcac c atg cac 357
 Met His
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 Leu Leu Gly Phe Phe Ser Val Ala Cys Ser Leu Leu Ala Ala Leu
 5 10 15

ctc ccg ggt cct cgc gag gcg ccc gcc gcc gcc gcc gcc ttc gag tcc 453
 Leu Pro Gly Pro Arg Glu Ala Pro Ala Ala Ala Ala Phe Glu Ser
 20 25 30

gga ctc gac ctc tcg gac gcg gag ccc gac gcg ggc gag gcc acg gct 501
 Gly Leu Asp Leu Ser Asp Ala Glu Pro Asp Ala Gly Glu Ala Thr Ala
 35 40 45 50

- 13 -

tat gca agc aaa gat ctg gag gag cag tta cgg tct gtg tcc agt gta	549
Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu Arg Ser Val Ser Ser Val	
55 60 65	
gat gaa ctc atg act gta ctc tac cca gaa tat tgg aaa atg tac aag	597
Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr Trp Lys Met Tyr Lys	
70 75 80	
tgt cag cta agg aaa gga ggc tgg caa cat aac aga gaa cag gcc aac	645
Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn Arg Glu Gln Ala Asn	
85 90 95	
ctc aac tca agg aca gaa gag act ata aaa ttt gct gca gca cat tat	693
Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala Ala Ala His Tyr	
100 105 110	
aat aca gag atc ttg aaa agt att gat aat gag tgg aga aag act caa	741
Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg Lys Thr Gln	
115 120 125 130	
tgc atg cca cgg gag gtg tgt ata gat gtg ggg aag gag ttt gga gtc	789
Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu Phe Gly Val	
135 140 145	
gcg aca aac acc ttc ttt aaa cct cca tgt gtg tcc gtc tac aga tgt	837
Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr Arg Cys	
150 155 160	
ggg ggt tgc tgc aat agt gag ggg ctg cag tgc atg aac acc agc acg	885
Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser Thr	
165 170 175	
agc tac ctc agc aag acg tta ttt gaa att aca gtg cct ctc tct caa	933
Ser Tyr Leu Ser Lys Thr Leu Phe Glu Ile Thr Val Pro Leu Ser Gln	
180 185 190	
ggc ccc aaa cca gta aca atc agt ttt gcc aat cac act tcc tgc cga	981
Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg	
195 200 205 210	
tgc atg tct aaa ctg gat gtt tac aga caa gtt cat tcc att att aga	1029
Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg	
215 220 225	
cgt tcc ctg cca gca aca cta cca cag tgt cag gca gcg aac aag acc	1077
Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr	
230 235 240	
tgc ccc acc aat tac atg tgg aat aat cac atc tgc aga tgc ctg gct	1125
Cys Pro Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys Leu Ala	
245 250 255	
cag gaa gat ttt atg ttt tcc tgc gat gct gga gat gac tca aca gat	1173
Gln Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp	
260 265 270	

- 14 -

gga ttc cat gac atc tgt gga cca aac aag gag ctg gat gaa gag acc 1221
 Gly Phe His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr
 275 280 285 290

tgt cag tgt gtc tgc aga gcg ggg ctt cgg cct gcc agc tgt gga ccc 1269
 Cys Gln Cys Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro
 295 300 305

cac aaa gaa cta gac aga aac tca tgc cag tgt gtc tgt aaa aac aaa 1317
 His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys
 310 315 320

ctc ttc ccc agc caa tgt ggg gcc aac cga gaa ttt gat gaa aac aca 1365
 Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr
 325 330 335

tgc cag tgt gta tgt aaa aga acc tgc ccc aga aat caa ccc cta aat 1413
 Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn
 340 345 350

cct gga aaa tgt gcc tgt gaa tgt aca gaa agt cca cag aaa tgc ttg 1461
 Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu
 355 360 365 370

tta aaa gga aag aag ttc cac cac caa aca tgc agc tgt tac aga cg 1509
 Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr Arg Arg
 375 380 385

cca tgt acg aac cgc cag aag gct tgt gag cca gga ttt tca tat agt 1557
 Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser Tyr Ser
 390 395 400

gaa gaa gtg tgt cgt tgt gtc cct tca tat tgg aaa aga cca caa atg 1605
 Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro Gln Met
 405 410 415

agc taa gattgtactg tttccagtt catcgatttt ctattatgga aaactgtggt 1661
 Ser
 420

gccacagtag aactgtctgt gaacagagag acccttgtgg gtccatgcta acaaagacaa 1721

aagtctgtct ttcctgaacc atgtggataa ctttacagaa atggactgga gctcatctgc 1781

aaaaggcctc ttgtaaagac tggttttctg ccaatgacca aacagccaag attttcctct 1841

tgtgatttct ttaaaagaat gactatataa tttatttcca ctaaaaatat tgtttctgca 1901

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caaaatatgt ttaaaataaa atgaaaattg tattat 1997

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<211> 419

<212> PRT

<213> Homo sapiens

- 15 -

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			20					25						30			
Glu	Ser	Gly	Leu	Asp	Leu	Ser	Asp	Ala	Glu	Pro	Asp	Ala	Gly	Glu	Ala		
		35					40					45					
Thr	Ala	Tyr	Ala	Ser	Lys	Asp	Leu	Glu	Glu	Gln	Leu	Arg	Ser	Val	Ser		
	50					55						60					
Ser	Val	Asp	Glu	Leu	Met	Thr	Val	Leu	Tyr	Pro	Glu	Tyr	Trp	Lys	Met		
	65				70					75					80		
Tyr	Lys	Cys	Gln	Leu	Arg	Lys	Gly	Gly	Trp	Gln	His	Asn	Arg	Glu	Gln		
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Ala	Asn	Leu	Asn	Ser	Arg	Thr	Glu	Glu	Thr	Ile	Lys	Phe	Ala	Ala	Ala		
		100						105					110				
His	Tyr	Asn	Thr	Glu	Ile	Leu	Lys	Ser	Ile	Asp	Asn	Glu	Trp	Arg	Lys		
		115					120					125					
Thr	Gln	Cys	Met	Pro	Arg	Glu	Val	Cys	Ile	Asp	Val	Gly	Lys	Glu	Phe		
	130					135					140						
Gly	Val	Ala	Thr	Asn	Thr	Phe	Phe	Lys	Pro	Pro	Cys	Val	Ser	Val	Tyr		
	145				150				155						160		
Arg	Cys	Gly	Gly	Cys	Cys	Asn	Ser	Glu	Gly	Leu	Gln	Cys	Met	Asn	Thr		
				165					170					175			
Ser	Thr	Ser	Tyr	Leu	Ser	Lys	Thr	Leu	Phe	Glu	Ile	Thr	Val	Pro	Leu		
		180						185					190				
Ser	Gln	Gly	Pro	Lys	Pro	Val	Thr	Ile	Ser	Phe	Ala	Asn	His	Thr	Ser		
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Cys	Arg	Cys	Met	Ser	Lys	Leu	Asp	Val	Tyr	Arg	Gln	Val	His	Ser	Ile		
	210					215					220						
Ile	Arg	Arg	Ser	Leu	Pro	Ala	Thr	Leu	Pro	Gln	Cys	Gln	Ala	Ala	Asn		
	225				230					235					240		
Lys	Thr	Cys	Pro	Thr	Asn	Tyr	Met	Trp	Asn	Asn	His	Ile	Cys	Arg	Cys		
				245					250					255			
Leu	Ala	Gln	Glu	Asp	Phe	Met	Phe	Ser	Ser	Asp	Ala	Gly	Asp	Asp	Ser		
		260						265					270				
Thr	Asp	Gly	Phe	His	Asp	Ile	Cys	Gly	Pro	Asn	Lys	Glu	Leu	Asp	Glu		
		275					280					285					
Glu	Thr	Cys	Gln	Cys	Val	Cys	Arg	Ala	Gly	Leu	Arg	Pro	Ala	Ser	Cys		
	290					295					300						

- 16 -

Gly Pro His Lys Glu Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys
 305 310 315 320

Asn Lys Leu Phe Pro Ser Gln Cys Gly Ala Asn Arg Glu Phe Asp Glu
 325 330 335

Asn Thr Cys Gln Cys Val Cys Lys Arg Thr Cys Pro Arg Asn Gln Pro
 340 345 350

Leu Asn Pro Gly Lys Cys Ala Cys Glu Cys Thr Glu Ser Pro Gln Lys
 355 360 365

Cys Leu Leu Lys Gly Lys Lys Phe His His Gln Thr Cys Ser Cys Tyr
 370 375 380

Arg Arg Pro Cys Thr Asn Arg Gln Lys Ala Cys Glu Pro Gly Phe Ser
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Tyr Ser Glu Glu Val Cys Arg Cys Val Pro Ser Tyr Trp Lys Arg Pro
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<211> 2029

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<213> Homo sapiens

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 cattttgatt tttttcatct ctctctcccc acccctaaga ttgtgcaaaa aaagcgtacc 240
 ttgcctaatt gaaataattt cattggattt tgatcagaac tgattatttg gttttctgtg 300
 tgaagttttg aggtttcaaa ctttccttct ggagaatgcc ttttgaaaca attttctcta 360
 gctgcctgat gtcaactgct tagtaatcag tggatattga aatattcaaa atg tac 416
 Met Tyr
 1

aga gag tgg gta gtg gtg aat gtt ttc atg atg ttg tac gtc cag ctg 464
 Arg Glu Trp Val Val Val Asn Val Phe Met Met Leu Tyr Val Gln Leu
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- 17 -

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Val Gln Gly Ser Ser Asn Glu His Gly Pro Val Lys Arg Ser Ser Gln	
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tcc aca ttg gaa cga tct gaa cag cag atc agg gct gct tct agt ttg	560
Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg Ala Ala Ser Ser Leu	
35 40 45 50	
gag gaa cta ctt cga att act cac tct gag gac tgg aag ctg tgg aga	608
Glu Glu Leu Leu Arg Ile Thr His Ser Glu Asp Trp Lys Leu Trp Arg	
55 60 65	
tgc agg ctg agg ctc aaa agt ttt acc agt atg gac tct cgc tca gca	656
Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met Asp Ser Arg Ser Ala	
70 75 80	
tcc cat cgg tcc act agg ttt gcg gca act ttc tat gac att gaa aca	704
Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Ile Glu Thr	
85 90 95	
cta aaa gtt ata gat gaa gaa tgg caa aga act cag tgc agc cct aga	752
Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln Cys Ser Pro Arg	
100 105 110	
gaa acg tgc gtg gag gtg gcc agt gag ctg ggg aag agt acc aac aca	800
Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys Ser Thr Asn Thr	
115 120 125 130	
ttc ttc aag ccc cct tgt gtg aac gtg ttc cga tgt ggt ggc tgt tgc	848
Phe Phe Lys Pro Cys Val Asn Val Phe Arg Cys Gly Gly Cys Cys	
135 140 145	
aat gaa gag agc ctt atc tgt atg aac acc agc acc tcg tac att tcc	896
Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser Thr Ser Tyr Ile Ser	
150 155 160	
aaa cag ctc ttt gag ata tca gtg cct ttg aca tca gta cct gaa tta	944
Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro Glu Leu	
165 170 175	
gtg cct gtt aaa gtt gcc aat cat aca ggt tgt aag tgc ttg cca aca	992
Val Pro Val Lys Val Ala Asn His Thr Gly Cys Lys Cys Leu Pro Thr	
180 185 190	
gcc ccc cgc cat cca tac tca att atc aga aga tcc atc cag atc cct	1040
Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln Ile Pro	
195 200 205 210	
gaa gaa gat cgc tgt tcc cat tcc aag aaa ctc tgt cct att gac atg	1088
Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu Cys Pro Ile Asp Met	
215 220 225	
cta tgg gat agc aac aaa tgt aaa tgt gtt ttg cag gag gaa aat cca	1136
Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu Gln Glu Glu Asn Pro	
230 235 240	

- 18 -

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 Leu Ala Gly Thr Glu Asp His Ser His Leu Gln Glu Pro Ala Leu Cys
 245 250 255

ggg cca cac atg atg ttt gac gaa gat cgt tgc gag tgt gtc tgt aaa 1232
 Gly Pro His Met Met Phe Asp Glu Asp Arg Cys Glu Cys Val Cys Lys
 260 265 270

aca cca tgt ccc aaa gat cta atc cag cac ccc aaa aac tgc agt tgc 1280
 Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro Lys Asn Cys Ser Cys
 275 280 285 290

ttt gag tgc aaa gaa agt ctg gag acc tgc tgc cag aag cac aag cta 1328
 Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys Gln Lys His Lys Leu
 295 300 305

ttt cac cca gac acc tgc agc tgt gag gac aga tgc ccc ttt cat acc 1376
 Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg Cys Pro Phe His Thr
 310 315 320

aga cca tgt gca agt ggc aaa aca gca tgt gca aag cat tgc cgc ttt 1424
 Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala Lys His Cys Arg Phe
 325 330 335

cca aag gag aaa agg gct gcc cag ggg ccc cac agc cga aag aat cct 1472
 Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His Ser Arg Lys Asn Pro
 340 345 350

tga ttcagcgttc caagttcccc atccctgtca tttttaacag catgctgctt 1525
 355

tgccaagtgt ctgtcactgt ttttttccca ggtgttaaaa aaaaaatcca ttttacacag 1585

caccacagtg aatccagacc aaccttccat tcacaccagc taaggagtcc ctggttcatt 1645

gatggatgtc ttctagctgc agatgcctct gcgcaccaag gaatggagag gaggggaccc 1705

atgtaatcct tttgtttagt tttgtttttg ttttttggtg aatgagaaag gtgtgctggt 1765

catggaatgg caggtgtcat atgactgatt actcagagca gatgaggaaa actgtagtct 1825

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tgattcgtat gatcagtact gactttctga ttactgtcca gcttatagtc ttccagttaa 1945

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 <212> PRT
 <213> Homo sapiens
 <400> 6

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 Ser Gln Ser Thr Leu Glu Arg Ser Glu Gln Gln Ile Arg Ala Ala Ser
 35 40 45
 Ser Leu Glu Glu Leu Leu Arg Ile Thr His Ser Glu Asp Trp Lys Leu
 50 55 60
 Trp Arg Cys Arg Leu Arg Leu Lys Ser Phe Thr Ser Met Asp Ser Arg
 65 70 75 80
 Ser Ala Ser His Arg Ser Thr Arg Phe Ala Ala Thr Phe Tyr Asp Ile
 85 90 95
 Glu Thr Leu Lys Val Ile Asp Glu Glu Trp Gln Arg Thr Gln Cys Ser
 100 105 110
 Pro Arg Glu Thr Cys Val Glu Val Ala Ser Glu Leu Gly Lys Ser Thr
 115 120 125
 Asn Thr Phe Phe Lys Pro Pro Cys Val Asn Val Phe Arg Cys Gly Gly
 130 135 140
 Cys Cys Asn Glu Glu Ser Leu Ile Cys Met Asn Thr Ser Thr Ser Tyr
 145 150 155 160
 Ile Ser Lys Gln Leu Phe Glu Ile Ser Val Pro Leu Thr Ser Val Pro
 165 170 175
 Glu Leu Val Pro Val Lys Val Ala Asn His Thr Gly Cys Lys Cys Leu
 180 185 190
 Pro Thr Ala Pro Arg His Pro Tyr Ser Ile Ile Arg Arg Ser Ile Gln
 195 200 205
 Ile Pro Glu Glu Asp Arg Cys Ser His Ser Lys Lys Leu Cys Pro Ile
 210 215 220
 Asp Met Leu Trp Asp Ser Asn Lys Cys Lys Cys Val Leu Gln Glu Glu
 225 230 235 240
 Asn Pro Leu Ala Gly Thr Glu Asp His Ser His Leu Gln Glu Pro Ala
 245 250 255
 Leu Cys Gly Pro His Met Met Phe Asp Glu Asp Arg Cys Glu Cys Val
 260 265 270
 Cys Lys Thr Pro Cys Pro Lys Asp Leu Ile Gln His Pro Lys Asn Cys
 275 280 285
 Ser Cys Phe Glu Cys Lys Glu Ser Leu Glu Thr Cys Cys Gln Lys His
 290 295 300

- 20 -

Lys Leu Phe His Pro Asp Thr Cys Ser Cys Glu Asp Arg Cys Pro Phe
 305 310 315 320

His Thr Arg Pro Cys Ala Ser Gly Lys Thr Ala Cys Ala Lys His Cys
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Arg Phe Pro Lys Glu Lys Arg Ala Ala Gln Gly Pro His Ser Arg Lys
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Asn Pro

<210> 7

<211> 17

<212> DNA

<213> Artificial Sequence

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 oligonucleotide

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17

<210> 8

<211> 18

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<223> Description of Artificial Sequence:
 oligonucleotide

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<210> 9

<211> 18

<212> DNA

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<400> 9

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18

<210> 10

<211> 18

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:oligonucleotide

<400> 10

gcccaggcct gtctactg

18

<210> 11

<211> 18

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:oligonucleotide

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18

<210> 12

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<212> DNA

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<223> Description of Artificial Sequence:oligonucleotide

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18

<210> 13

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<223> Description of Artificial Sequence:oligonucleotide

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18

<210> 14

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<212> DNA

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<223> Description of Artificial Sequence:oligonucleotide

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18

<210> 15

<211> 21

<212> DNA

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<223> Description of Artificial Sequence:oligonucleotide

<400> 15

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21

<210> 16

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<212> DNA

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<223> Description of Artificial Sequence:oligonucleotide

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18

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<223> Description of Artificial Sequence:oligonucleotide

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cctgagtatc tcccgcgtcg c

21

<210> 18

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<220>

<223> Description of Artificial Sequence:oligonucleotide

<400> 18

ggcgcctccc tgtaccctgg g

21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06133

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06133

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12Q 1/68; A61K 38/00, 38/19, 48/00; C07H 21/02, 21/04; C12N 15/11; C12P 19/34; G01N 27/26

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 91.2; 424/85.1; 514/2, 44, 536/23.1; 536/24, 33, 24, 31, 24.3; 204/450

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-10, drawn to methods of screening humans for lymphatic disorders by detecting mutations.

Group II, claim(s) 11-12, drawn to methods of treatment.

Group III, claim(s) 13-17, drawn to oligonucleotides.

Group IV, claim(s) 18-21, drawn to vectors and host cells.

Group V, claim(s) 22-25, drawn to methods of identifying compounds.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The mutations in the nucleic acid sequence of VEGFR-3 associated with disease, which is considered the technical feature linking all the claims, were well known in the art at the time of filing as taught by Boulton (1994, Genomics, Vol. 19, pages 425-432). It would have been obvious to detect mutations in the VEGFR-3 to screen for lymphatic disorder, replace the VEGFR-3 using gene therapy, make oligonucleotides with the mutated VEGFR-3, make vectors and host cells with the mutated VEGFR-3 or identify compounds having an effect on cells with mutated VEGFR-3.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06133

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2; 424/85.1; 514/2, 44, 536/23.1; 536/24, 33, 24, 31, 24.3; 204/450

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
noneElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, Caplus, biosis, medline**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THOMPSON et al. The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. Dev. Biology. 15 May 1998, Vol. 197, No. 2, pages 248-269, see entire article.	13-25
Y	FERRELL et al. Hereditary lymphedema: evidence for linkage and genetic heterogeneity. Hum. Mol. Genetics. December 1998, Vol. 7, No. 13, pages 2073-2078, see entire article.	1-25
Y	FOURNIER et al. Mutation in tyrosine residue 1337 abrogates ligand-dependent transforming capacity of the FLT4 receptor. Oncogene. 1995, Vol. 11, No. 5, pages 921-931, see entire article.	1-10

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

24 MAY 1999

Date of mailing of the international search report

22 JUN 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06133

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FOURNIER et al. Role of tyrosine residues and protein interaction domains of SHC adaptor in VEGF receptor 3 signaling. Oncogene. 14 January 1999, Vol. 18, No. 2, pages 507-514, see entire article.	1-25
Y	WITTE et al. Phenotypic and genotypic hetherogeneity in familial Milroy lymphedema. Lymphology. December 1998, Vol. 31, No. 4, pages 145-155, see entire article.	1-10
Y	BOULTWOOD et al. Molecular mapping of uncharacteristically small 5q deletions in two patients with the 5q- syndrome: delineation of the critical region on 5q and identification of a 5q-breakpoint. Genomic. 1994, Vol. 19, No. 3, pages 425-432, see entire article.	1-10

1 / 5

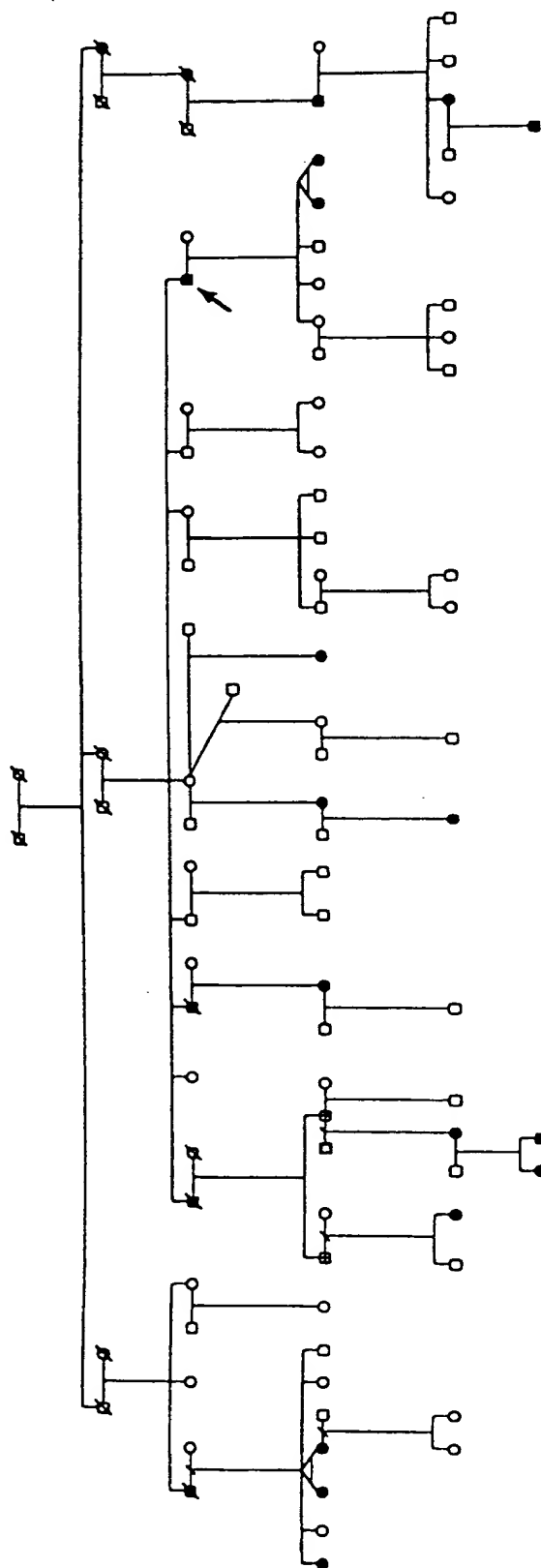


Fig. 1A

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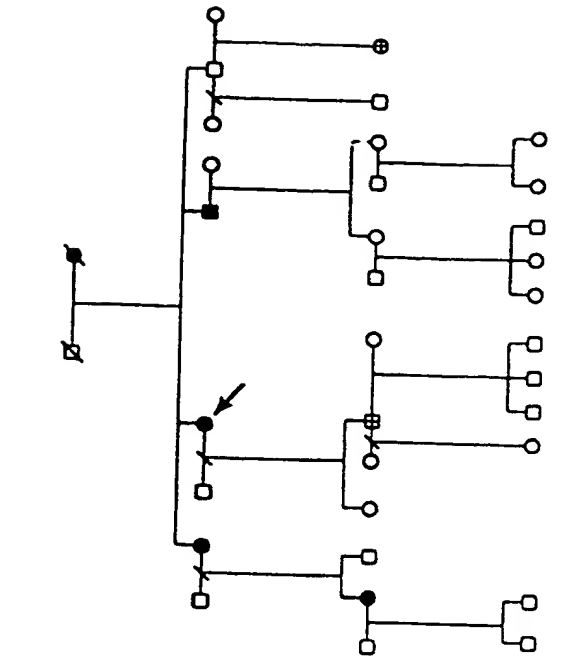


Fig. 1C

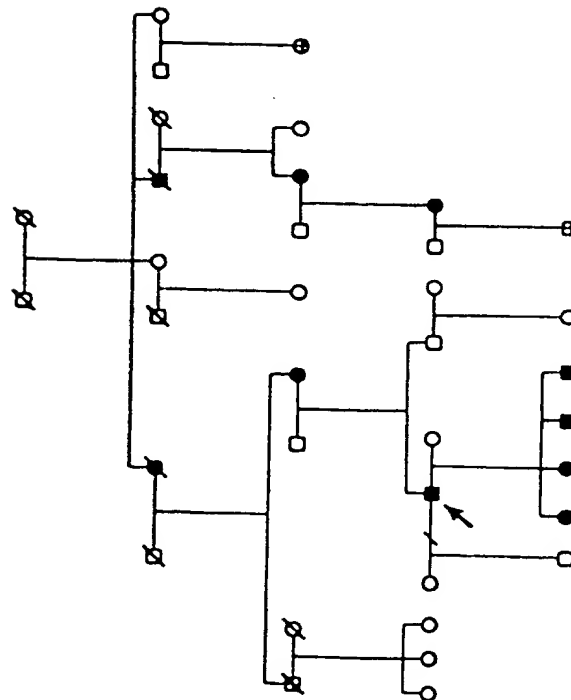


Fig. 1B

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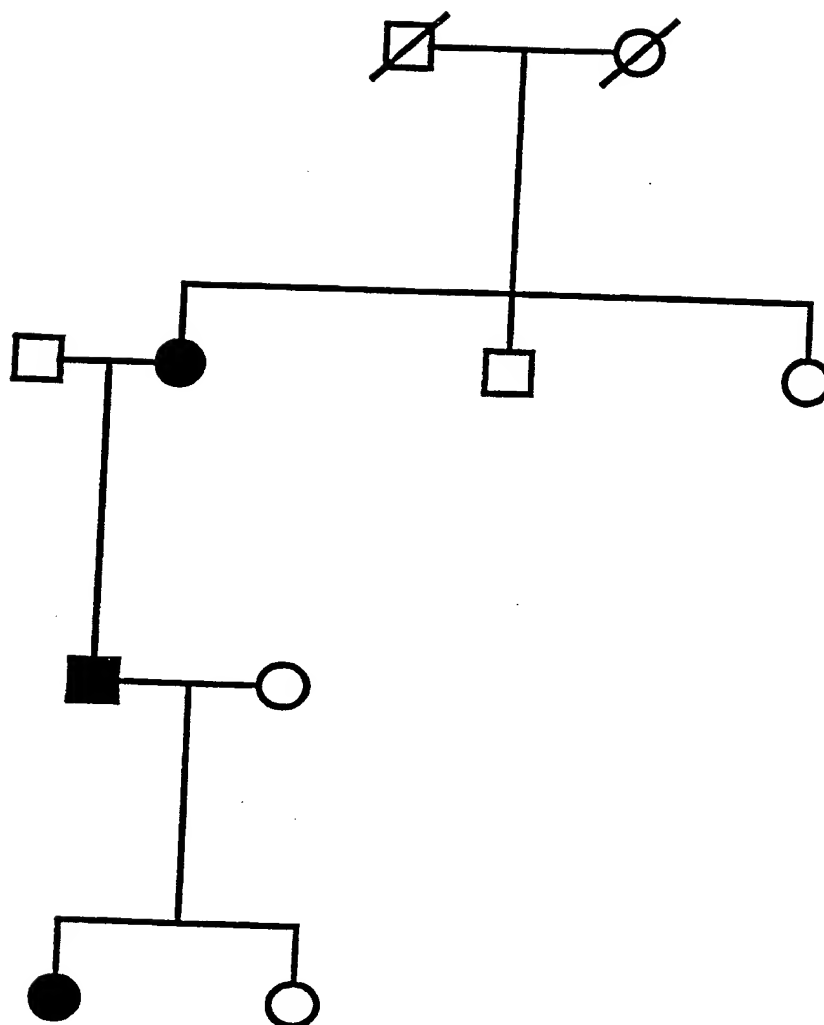


Fig. 1F

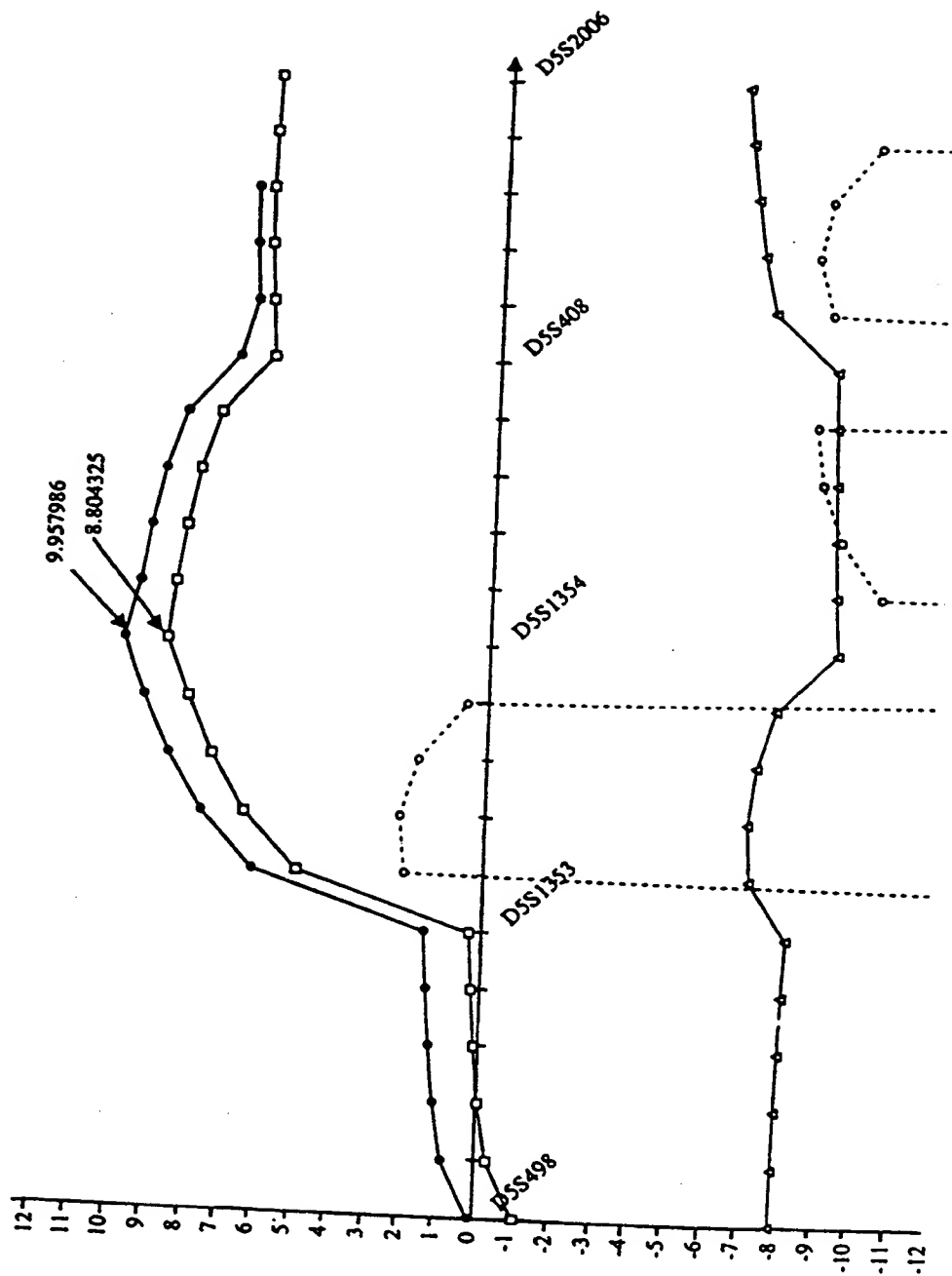


Fig. 2

